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by

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Declaration

Except where explicitly stated, this thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration. This thesis has not been submitted in whole or part to any other university.

Abstract

The Wilms' tumour antigen 1 (WT1) is a transcription factor which is over-expressed in several leukaemias and solid tumours. Currently, there is limited information about the expression and immunogenicity of WT1 in prostate cancer. I used immunohistochemistry to analyse WT1 expression in prostate cancer samples and tetramers to detect WT1-specific T cell responses in the peripheral blood. 39% of cancer samples showed nuclear and cytoplasmic staining for WT1, whereas the staining of all normal prostate tissue was limited to the cytosol ($p<0.03$). Furthermore, WT1-specific T cells which bound tetramer were detectable in the peripheral blood of 20/38 (53%) prostate cancer patients, *ex vivo*. Although these T cells did not expand in 20/20 patients using peptide stimulation with IL2/IL7, a population of WT1-specific CTL accumulated in 3/8 patients following culture with IL15.

T cell receptor (TCR) gene transfer is a strategy to circumvent possible impairment of autologous T cell responses against tumour associated antigens such as WT1. However, exogenous and endogenous TCRs are in competition for CD3 chains, which may reduce the expression of the introduced TCR and impair the antigen specific function of transduced T cells. I developed an approach to improve the expression of exogenous TCR chains by co-transducing TCR genes together with the genes encoding the CD3 complex. Transfer of CD3 and TCR genes into primary T cells resulted in up to a 5 fold increase in TCR expression and up to a 9 fold increase in tetramer binding compared to that seen after transfer of TCR genes alone. Furthermore, T cells expressing high levels of TCR/CD3 demonstrated increased sensitivity. This data indicates that the efficacy of TCR expression, and the effector function of gene modified T cells, is substantially enhanced by the co-transfer of CD3 genes.

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AIRE	autoimmune regulator
APC	antigen presenting cell
APC	allophcocyanin
bp	base pairs
cDNA	complementary DNA
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
EGFR	epidermal growth factor receptor
ERAAP	endoplasmic reticulum aminopeptidase associated with antigen processing
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GvL	graft-versus-leukaemia
HLA	human leukocyte antigen
HPV	human papilloma virus
HSP	heat shock protein
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine based activation motif
LAT	linker of activated T cells
M	Molar
m	mill (10^{-3})
μ	micro(10^{-6})
MCA	3-methylcholanthrene
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
mRNA	messenger RNA
n	nano(10^{-9})
NK	natural killer (cell)
p	pico(10^{-12})

PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PRR	pattern recognition receptor
PTLD	post transplant lymphoproliferative disease
RAG	recombinase-activating gene
RECIST	response evaluation criteria in solid tumours
RNA	ribonucleic acid
RPMI	Rockwell Park Memorial Institute 1640 medium
SMAC	supramolecular adhesion complex
STAT	signal transducer and activator of transcription
TAA	tumour-associated antigen
TAP	transporter associated with antigen processing
T cell	thymus derived lymphocyte
TCR	T cell receptor
TGF	transforming growth factor
TIL	tumour infiltrating lymphocyte
TLR	Toll like receptor
Th	T helper (cell)
Treg	Regulatory T cell
TSA	tumour-specific antigen
U	units
VEGF	vascular endothelial growth factor
WT1	Wilms' tumour 1

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JK

Chapter 1: General Introduction

1.1 Tumour immunotherapy

Cancer is a common disease which will affect 1 in 3 people during their lifetime. As the population continues to age, the incidence of cancer continues to rise. While surgery and radiotherapy are effective treatments for localised disease, a large proportion of patients present with metastatic disease. Traditional chemotherapeutic agents exert their effects by interfering with cell division and work on the premise that cancer cells will be dividing more quickly than healthy cells. Hence, chemotherapy also has predictable side effects on dividing cells in the bone marrow, skin, gastrointestinal tract epithelium and hair follicles. Although chemotherapy has been shown to improve survival in many different tumours, in the vast majority of cases chemotherapy for metastatic disease remains a palliative treatment. Thus, the ratio of the inherent risks of the treatment versus the potential benefit to patients is always a major consideration, given the toxicities associated with conventional treatment.

The focus of current cancer research is on providing targeted anti tumour therapy which can distinguish between malignant and normal cells, therefore reducing the toxic side effect profile commonly experienced with traditional chemotherapeutic agents. Antigen specific immunotherapy is an example of a targeted therapy which aims to reduce the risks associated with treatment while increasing the benefit to patients.

The goal of tumour immunotherapy is to target tumours by providing either active or passive immunity against malignancies. In passive immunotherapy, the effectors of the immune response (e.g. antibodies or T cells) are directly transferred into patients. Active immunotherapy, of which vaccination is an example, requires the patient to mount an immune response against cancer antigens.

Early attempts at cancer immunotherapy focused on cytokines such as interleukin-2 (IL2) that activate both innate and adaptive immune responses in a non antigen specific manner. However, cytokine therapy is associated with significant systemic side effects. Advances in scientific knowledge and technology have led to the development of antibody and T cell based therapies which utilise the unique specificity of immune receptors to target selected antigens on tumour cells. This reduces their systemic side effect profile compared to cytokine therapy.

1.1.1 Tumour immunosurveillance

The increased incidence of certain tumours in immunosuprressed patients compared to age matched controls is well established, for example: non Hodgkin's lymphoma, Kaposi's sarcoma, cervical carcinoma and skin cancers (1-3). The concept that the immune system could play a role in detecting and preventing the development of tumours was first described by Erlich in 1909 (4). He proposed the concept that spontaneously arising tumours could be recognised and destroyed by cells of the immune system. The "cancer immunosurveillance hypothesis" was reintroduced decades later by Burnet and Thomas. They suggested that the mammalian immune system had evolved to recognise mutations which contribute towards the malignant phenotype of cancer cells, and that the immune system was responsible for the destruction of malignancies in mammals that do not present clinically (5-7). However, the experiments performed to investigate the hypothesis remained inconclusive. More recently, new experimental models were developed to support the concept of immunosurveillance.

It has been shown that there is an increased incidence of spontaneous and carcinogen induced tumours in several murine models where elements of the immune system are defective (8). The definitive experiments were performed in Rag deficient mice (Rag-/-), which do not produce any T or B lymphocytes. This model was used to study the effect of immunodeficiency on tumour development. It was shown that Rag-/- mice developed a greater number of tumours more quickly and more frequently in response to a chemical carcinogen (MCA) than wild-type control mice. IFNγ insensitive mice (lacking either the IFNγ receptor or STAT1, a transcription factor which plays a crucial role in IFNα/β and IFNγ receptor signalling) also developed more tumours than wild type controls. The same group also demonstrated that spontaneous adenocarcinoma development was significantly more frequent in Rag-/- mice and in Rag-/- mice which also had an IFN signalling deficit, compared with wild type mice (9). This data supported the idea that the immune system can recognise and destroy cancer cells based on differences between normal and cancerous cells.

However, this concept is at odds with the fact that cancers commonly develop in immunocompetent hosts. This may be a result of immune surveillance putting selective pressure on tumour cells to develop ways of evading the immune system and becoming less immunogenic, a process described as cancer immunoediting (8;10).

1.1.2 Cancer immunoediting

Normal cells are under tight regulation of cell division, growth and controlled cell death (apoptosis). By developing mutations in the tumour suppressor genes or oncogenes which control these processes, normal cells develop a malignant phenotype and hence the ability to divide and grow inappropriately. The hallmarks of cancer are: self sufficiency in growth signals, ignoring growth inhibition signals, evasion of apoptosis, replicative potential, angiogenesis and tissue invasion (11). It has been suggested that immune evasion is the 7th hallmark of cancer (8). This has implications for immunotherapy, as tumours that develop will, by definition, have escaped control by naturally occurring immune responses.

The process of cancer immunoediting has been proposed to involve 3 phases: elimination, equilibrium and escape. The elimination phase involves transformed cells being recognised and killed by cells of both the innate and adaptive immune systems. The equilibrium phase describes the balance between tumour cell killing and survival, resulting in the tumour being contained, but not eliminated. During this phase there is selective pressure on tumour cells to develop ways of evading or inhibiting immune responses, resulting in the growth of tumour escape variants. There are a number of mechanisms by which tumours can become less immunogenic. These include the loss of target antigen expression or downregulation of MHC Class 1 molecules, the secretion of immunosuppressive cytokines such as IL-10 and TGFβ.

However, the immunosurveillance theory remains controversial. Using a murine sporadic tumour model, Willimsky and Blankenstein demonstrated that although specific immune responses against developing tumours did arise, they did not curb the tumour's growth, and that in fact the sporadic tumours induced T cell tolerance. They also showed that the tumours which developed in the presence of a normal immune system remained immunogenic and were not escape variants (12). Thus, in the absence of consistently reproducible data supporting it, the concepts of immunosurveillance and immunoediting continue to be contentious topics for immunologists.

1.2 Immunotherapy and the cells of the immune system

The immune system can be divided into the innate and adaptive immune systems, both of which can be manipulated for cancer immunotherapy. I will deal with each in turn.

1.2.1 Innate immune system

The innate immune system is highly conserved between species and forms a crucial first line of defence against infection. It consists mainly of myeloid lineage cells: macrophages, neutrophils, eosinophils and mast cells, in addition to natural killer (NK) cells, dendritic cells (DC) and the complement pathway. Once activated, cells of the innate immune system can kill their targets or secrete cytokines to recruit other effectors of the innate and adaptive immune systems to an infected area.

The innate immune system tends to recognise targets commonly found on many pathogens, but distinguishable from host molecules: pathogen-associated molecular patterns (PAMPs). Toll like receptors (TLR) are a type of pattern recognition receptor (PRR) present on many cells of the innate immune system. They form a receptor superfamily with the Interleukin-1 receptors and recognise a range of PAMPs such as bacterial or viral DNA, lipopolysaccharide (LPS), which is a component of Gram negative bacterial cell walls, and mannose – a carbohydrate which forms part of the cell wall of many pathogens. TLR ligands have been successfully used as immunomodulators in preclinical and clinical trials. For example, imiquimod, a TLR7/8 ligand, has been shown to be effective in the treatment of basal cell carcinoma in a randomized, blinded, multi-centre phase III clinical trial (13). Melanoma patients vaccinated with a melanoma antigen plus a synthetic TLR-9 analogue exhibited melan A specific T cell responses an order of magnitude higher than those vaccinated in the absence of the TLR ligand (14).

1.3 The adaptive immune system

The adaptive immune system is common to vertebrates and is thought to have evolved in order to deal with viral infections. It is antigen specific, the unique specificity being conferred by the T or B cell surface receptor, and also results in the formation of a memory response. Antigen specific immunotherapy seeks to direct T or B cell receptors (antibodies) against tumour antigens, the aim being that targeted therapy should reduce the side effect profile seen with conventional therapy.

Widespread clinical success has been seen with monoclonal antibodies directed against a number of targets including HER2, CD20, vascular endothelial growth factor (VEGF) and epidermal growth factor receptors (EGFR). This class of cancer therapeutics continues to grow rapidly, with a far less toxic side effect profile than conventional chemotherapy and radiotherapy (15-19).

However, antibody therapy has a number of limitations. Firstly, since a memory response is not generated, repeated antibody infusions are required. Secondly, because some monoclonal antibodies retain a small murine component, they are themselves potentially immunogenic, which may cause problems with repeated administration. Thirdly, antibodies can only recognise specific proteins which are present on the cell surface, which limits the range of available targets. Intracellular proteins are broken down into a large number of peptides, which are expressed on the cell surface in association with a major histocompatibility molecule (MHC). These peptide-MHC complexes are recognised by the T cell receptor (TCR), which is able to recognise a far wider range of antigens than antibodies, and therefore a much larger number of potential immunotherapy targets.

1.3.1 T cell overview

T lymphocytes originate in the bone marrow and differentiate in the thymus. 95% of T cells express a T cell receptor (TCR) composed of an α and a β chain. αβ T cells can be further subdivided according to expression of the co-receptors CD4 or CD8. CD8 T cells recognise peptides derived from intracellular proteins presented in the context of MHC Class I molecules and are able to kill infected cells and tumour cells. CD8 T cells are subdivided into Tc1 and Tc2 cells depending on their cytokine secretion profile. CD4 cells recognise peptides from exogenous proteins presented in the context of MHC Class II molecules, and can also be further sub-classified according to the cytokines they secrete.

Approximately 5% of T cells express a TCR comprised of a gamma and a delta chain: γδ T cells, of which there are several subsets. They have been described as a bridge between the innate and adaptive immune systems, and encompass features of both. Similarly to αβ T cells, a subset of γδ T cells can develop in the thymus, rearrange TCR genes to produce junctional diversity and are found in the lymphoid tissue (20). In addition, γδ T cells can be described as either naïve (CD45RA) or memory (CD45RO) in terms of both function and phenotype (21;22).

A second subset of γδ T cells are most prevalent in epithelial tissues such as the gut, lung and reproductive tract, where they display receptors of limited diversity. Unlike αβ T cells, γδ T cells do not generally recognise peptide antigens presented by MHC molecules. γδ T cells can recognise self proteins which are upregulated as a result of infection or cellular stress, such as heat shock proteins, MIC-A and MIC-B (23). γδ T cells may also act via pattern recognition receptors similar to those seen on cells of the innate immune system, and have been reported to express TLR2 and NKG2D (24) Although the range of molecules which activate γδ T cells has not been fully characterised, all Vγ9/Vδ2 T cells recognize the microbial compound 4-hydroxy-3-methyl-but-2-enylprypophosphate (HMB-PP), which is an essential metabolite in most pathogenic bacteria.

1.4 Antigen recognition

Whereas B cells recognise intact molecules expressed on the surface of a cell, T cells recognise peptides in association with an MHC molecule. One advantage of this is that, because the peptides are derived from the cell interior, T cell receptors are able to recognise cells infected with a virus even when the virus is not expressing full length proteins on the cell surface. Another advantage is that a protein on the cell surface may be folded in such a way that certain epitopes are not exposed. Breaking a protein into peptide epitopes vastly increases the number of targets available for recognition by TCRs. There are separate pathways by which antigens originating either inside or outside the cell are processed.

1.4.1 Class I antigen processing pathway

CD8 T cells recognise peptides presented in association with self MHC Class I molecules, which are present on all nucleated cells. The MHC Class I molecule is a heterodimer consisting of an α chain which is non-covalently linked to a β-microglobulin chain. The α chain consists of three extracellular domains: α1 and α2 are folded together to form a cleft which forms the peptide binding domain, while α3 resembles an immunoglobulin constant domain which spans the cell membrane.

The peptides presented by MHC Class I molecules originate in the cell cytoplasm, and include viral, bacterial and tumour derived as well as self proteins. It was initially thought that the main source of antigenic peptides was the breakdown of proteins at the end of their lifespan (25). However, peptides are also produced from the breakdown of defective products of protein synthesis (or defective ribosomal products: DRiPs) which are the result of mistranslations, misfolding, truncations or incorrect post translational modification (26). One advantage of this is that changes in

intracellular gene expression, for example as a result of viral infection, will rapidly be translated into peptide expression on the cell surface (27;28). A large, multicatalytic protease complex in the cytoplasm called the proteasome is responsible for cleaving proteins into peptides. Proteasomes are highly conserved between species and are present in all eukaryotes. They are comprised of 28 subunits which form a cylindrical structure: 4 stacked rings each of 7 subunits, with the active sites of the proteolytic subunits lining the core of the cylinder. Inhibitors of the proteolytic activity of the proteasome inhibit antigen presentation by Class I molecules (29). The main function of the proteasome is the ubiquitin-dependent degradation of cytosolic proteins; experimentally tagging proteins with ubiquitin results in their peptides being more efficiently presented by MHC Class I molecules (30;31).

Heat shock proteins (HSP) are also thought to play an important role in transferring peptides from the cytosol onto MHC Class I molecules within the endoplasmic reticulum (ER). HSP ordinarily make up approximately 5% of normal intracellular proteins, but this figure may increase to over 15% when the cell is under stress (for example: at high temperatures). Initial studies on HSP gp96 showed that animals which were immunised with gp96 preparations isolated from certain tumours were then protected against challenge by the same, but not other, tumours. Subsequent studies suggested that this was due to the ability of the gp96 chaperone protein to bind to a pool of intracellular peptides. Similar results were observed with two further cytoplasmic proteins, HSP70 and HSP90 (32). However, it is not clear whether the tumour rejection induced by HSP is peptide specific, or independent of the bound peptide and instead mediated by the innate immune system (33). The nature of processed peptides that associate with these chaperones is not well understood, nor is the role the peptide/chaperones play in the antigen-processing pathway. It remains unclear whether these chaperones act as transporters between different steps of the antigen-processing pathway (34) or whether they act as peptide sinks (35).

Peptides are loaded onto MHC molecules which are first assembled in the endoplasmic reticulum (ER). Since they are only stable with peptide occupying their peptide binding groove, a number of chaperone proteins play an important role in stabilising MHC molecules until this occurs. During assembly, partially folded MHC molecules are bound to the chaperone protein calnexin until the Class I α chain associates with β2 microglobulin. The calnexin then dissociates and the MHC complex binds to a complex of chaperone proteins including calreticulin, thiol-oxidoreductase Erp57 and tapasin, which associates with the transporter associated with antigen processing: TAP. TAP consists of a heterodimer formed by TAP1 and

TAP2, which spans the ER membrane and plays a crucial role in allowing peptides from the cytosol to enter the ER lumen. This is an ATP dependent process; TAP is a member of the ATP-binding cassette (ABC) transporter family (36). TAP transporter proteins most efficiently transport peptides which are 8 to 13 amino acids long: the appropriate length to fit in the Class I MHC groove.

Longer peptides which are transported into the ER are then trimmed by the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP). This enzyme removes residues from the amino end of peptides which are too long to fit into the MHC Class I peptide binding groove. Upon binding of a peptide in the MHC groove, the MHC molecule dissociates from its chaperone proteins (the TAP:tapasin:catreliculin:Erp57 complex) and is transported to the cell surface (37).

Role of interferons in Class I antigen processing

Interferon production, induced primarily by viral infections, exerts a strong influence on the Class I processing pathway by increasing the efficiency of antigen presentation through an increase in MHC Class I expression. In addition, while the proteasome exists in a constitutive form in all cells, IFNγ causes a change in the proteasome by inducing expression of subunits b1i, b2i and b5i, which substitute for constitutively expressed subunits, resulting in the production of the immunoproteasome. This cleaves peptides after hydrophobic residues with increased frequency, thus producing peptides with a preferred structure for binding both TAP and MHC class I molecules (38-40).

IFNγ also induces PA28 α and β, which make up the PA28 proteasome-activator molecule. PA28 is a six or seven membered ring which binds to the ends of the cyclindrical proteasome structure, opening up the ends to increase the rate at which both proteins enter and peptides can be released. IFN also upregulates the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP). In addition, interferons increase the expression of both Class I and Class II MHC molecules. IFNγ can also induce Class II expression on cells that do not constitutively express it (41;42)

1.4.2 Class II antigen processing

The MHC Class II molecule is composed of an α and β chain; transmembrane glycoproteins which are non-covalently linked. The peptide binding domain contains the greatest polymorphic diversity and is formed by the α1 and β1 subunits. Class II molecules present antigen to CD4 T cells, and are generally found on cells of the

immune system: thymic stromal cells, T cells in humans, B cells, DCs and macrophages. Class II molecules display peptides derived from the cell exterior which have been phagocytosed, as well as proteins derived from pathogens which replicate within intracellular vesicles. Endocytic vesicles, having become more acidic as they progressively move further from the cell exterior, fuse with lysosomes. These contain proteases including cathepsins which are activated at low pH and break down proteins into peptides which are typically 13-17 amino acids in length (43-45).

Class II molecules are synthesized in the ER where they associate with a trimeric MHC Class II-associated invariant chain complex. This occupies the peptide binding groove to prevent peptides within the ER from binding, and also targets Class II molecules to the endosomal compartment (46-50). Here the invariant chain is cleaved several times by proteases such as cathepsin S to release the Class II molecule from the invariant chain, leaving only a short peptide fragment still bound: CLIP (class II associated invariant chain peptide).

Specialised Class II-like molecules within endosomes then catalyse the peptide loading of the MHC Class II peptide binding groove. HLA-DM is comprised of an α and β chain which closely resemble those of Class II molecules, but differs in that it is not expressed on the cell surface. HLA-DM binds to MHC Class II:CLIP complexes and catalyses the release of CLIP and the binding of antigenic peptides. HLA-DM also binds to MHC Class II:peptide complexes, removing weakly bound peptides and allowing them to be replaced by other peptides. HLA-DM is negatively regulated by a further atypical MHC Class II molecule, HLA-DO, which is also found within vesicles rather than on the cell surface. HLA-DO binds to HLA-DM and prevents it from releasing CLIP from Class II molecules, thus preventing peptides from binding. In inflammation, the release of IFN-γ from NK cells and T cells upregulates expression of HLA-DM, but not its negative regulator, HLA-DO, the net result of which is increased antigen presentation (51-53).

1.4.3 Cross presentation

In the main, antigens within the cytosol are presented by Class I molecules to CD8 T cells and antigens from outside the cells are presented by Class II molecules to CD4 cells. The exception to this is when antigen presenting cells take up external antigen, yet present it on Class I molecules: cross presentation (54;55). Cross presentation is required to induce a CD8 T cell response against a virus which does not infect professional antigen presenting cells. The mechanism has yet to be elucidated, but may involve antigens being transferred from phagosomes into the cytosol before

cleavage by cytosolic proteasomes and transport into the ER via TAP (56-58) Cross presentation has been demonstrated by a number of groups who have suggested that it plays an important role in presenting antigens derived from tumours as well as viruses (59;60)

1.5 Choosing a target for cancer immunotherapy

The ideal anti-cancer agent would specifically target tumours without having deleterious effects on normal cells. An appropriate antigen target for immunotherapy is therefore one which is either only expressed on tumours, or expressed at very high levels relative to normal tissues. In addition, those cancers which develop in association with chronic viral infections may express viral antigens.

In theory, one would expect tumour specific antigens (TSA) to be better targets, since they are only expressed on cancer cells. TSA may arise as a result of gene mutations and translocations, such as the bcr/abl tyrosine kinase seen in chronic myeloid leukaemia (61) or β-catenin in melanoma (62). However, a drawback of using TSA as targets is that if patients harbour different mutations, they would need individual therapies recognising each individual target. A good target for cancer immunotherapy is therefore also one which is present on the tumours of multiple individuals.

TSA are often not well recognised by T cells. This is due to the peptide products of TSA being poorly presented by host Class I molecules. This may be because they have a poor binding affinity for self MHC molecules, in which case they will be out-competed by host self peptides for association with MHC molecules and presentation on the cell surface.

The majority of cancer immunotherapy targets identified to date are normal cellular proteins which are expressed at elevated levels in tumours: tumour associated antigens (TAA). The aim is to exploit the difference in the surface expression level of the target antigen on tumours compared to that on normal tissue. Expression in normal tissues may also be restricted, as is the case with testis cancer antigens. Since TAA are non mutated proteins they are not specific to individual patients, therefore strategies targeting a specific TAA can be used for all patients whose cancer over-expresses the target antigen. For example, MART-1 is over-expressd on the majority of melanomas and the Wilm's Tumour Antigen 1 (WT1) is over expressed on a number of different tumours including cancers of the colon, breast, lung and ovary. An immunotherapeutic strategy which targets WT1 can therefore be used against multiple tumour types.

TAA can be broadly divided into three groups, the first of which is the cancer testes antigens, which are usually only expressed by the germline cells of the testes. Examples include MAGE-1 and NY-ESO, which are upregulated in a number of malignancies including melanoma, breast and oesophageal cancer (63;64). These were originally thought to be tumour specific, since germline cells do not express MHC Class I and therefore would not express these antigens to the immune system. However, MAGE-1 and NY-ESO have been found to be expressed by medullary thymic epithelial cells, albeit at lower levels than in the testis, meaning that they are still likely to be subject to tolerance mechanisms (65).

The next group consists of the differentiation antigens. These are tissue specific antigens which are expressed by normal cells, but over-expressed when these cells become malignant. The melanoma differentiation antigens are the best described antigens of this group and include MART-1, tyrosinase and gp100, whose expression is limited to normal melanocytes and malignant melanomas. (66-68) Another example would be the prostate specific antigen (PSA), which is expressed on normal prostate cells and is over-expressed in prostate cancer.

The third TAA group consists of antigens which are expressed in a range of normal tissues, but which are over-expressed in malignant cells. Rather than being tissue specific, these TAA are expressed by a wide range of tissues and therefore malignancies. Examples include proteins derived from HER2/neu, cyclin d1, muc1 and telomerase reverse transcriptase. Some TAA are expressed in the majority of normal cells, such as p53 and mdm2, while others, such as WT1, have a limited expression pattern in normal tissue. The great advantage of using this group of targets for immunotherapy is that if multiple tumour types express the same target, a greater number of patients could potentially benefit.

However, there are two significant drawbacks to using TAA as targets for immunotherapy. The first is that tolerance mechanisms may affect the avidity of immune responses generated against them. The second is the risk of auto-immune damage to healthy tissues which also express the self antigen. I will discuss both of these issues in more detail below.

1.5.1 TAA and tolerance

Tolerance mechanisms exist to prevent autoimmunity. Potentially harmful, self-reactive T cells are deleted or rendered unresponsive. Tolerance can either be central, in the thymus, or peripheral.

1.5.2 Central tolerance

During normal T cell development, immature T cell precursors from the bone marrow enter the thymus where they mature and proceed to a two stage selection process. Positive selection takes place in the cortex of the thymus. TCRs, having been generated by a complex process of gene rearrangement, are then 'tested' against thymic cortical epithelial cells (which express MHC Class I and II molecules) to ensure that T cells which proceed are capable of recognising peptides in association with self MHC. Approximately 90% of T cells entering the thymus express a TCR which does not interact with self MHC, and subsequently die by neglect (69;70).

The positively selected cells are then 'tested' again against thymic antigen presenting cells in the medulla which present self peptides. T cells which recognise self peptide/MHC combinations with high avidity - and would therefore potentially be autoreactive – are induced to undergo apoptosis (71). Approximately half of all T cells which are positively selected are then deleted by this process of clonal deletion, or negative selection (72).

However, not all self peptides are naturally presented by thymic cells, so a mechanism exists to ensure that T cells which recognise peripherally expressed self antigens with high avidity are also subject to negative selection. The autoimmune regulator (AIRE) transcription factor controls the expression of peripheral tissue genes in thymic medullary cells. Deletion of AIRE is associated with reduced transcription of peripheral antigen genes and the development of autoimmune disease, highlighting the importance of central tolerance in preventing autoimmunity. (73-75).

The combination of positive selection and negative selection ensures that the T cells which leave the thymus are able to recognise self MHC, but not self peptides with high avidity. However, central tolerance is incomplete, as self reactive T cells can be found in the periphery. Studies have shown that T cells can escape central tolerance by downregulating co-receptors or by expressing two TCRs simultaneously, and T cells which recognise self antigens with low avidity can also escape central tolerance (76-78). A consequence of this for tumour immunology is that only T cells which recognise TAA with low avidity will be allowed to persist in the circulation, and their low avidity may limit their ability to kill tumour cells.

1.5.3 Peripheral tolerance

As a further safeguard to deal with autoreactive T cells which escape central tolerance, peripheral tolerance mechanisms also exist. There are a number of explanations for self reactive T cells being able to evade central tolerance. Despite the presence of the AIRE gene, not all peripheral antigens are expressed in the thymus, or they may be presented at too low a level, or on too few cells, to induce clonal deletion.

Peripheral tolerance may occur by a number of mechanisms. Self reactive T cells may be "ignorant" of their self antigen: the antigen may be expressed in anatomical sites that the T cell cannot access, or may be expressed at too low a level to activate the T cell (79;80). Alternatively, self reactive T cells may be deleted in the periphery (81) or rendered anergic. Anergic T cells are unresponsive: they do not produce IL-2 when the TCR binds its cognate antigen and are unable to proliferate. Anergy can result from a TCR binding its cognate antigen in the absence of a second costimulatory signal, or in the presence of CTLA-4 binding to CD80 or CD86 (82;83).

Regulatory T cells

Regulatory T cells (Tregs) make up approximately 5-10% of CD4 T cells and are characterised by the expression of CD25 and the transcription factor FoxP3. They are a subset of T cells of thymic origin which actively suppress activation of the immune system via cell to cell contact, and prevent autoimmune disease resulting from pathological self-reactivity (84;85).

The critical role regulatory T cells play within the immune system is evidenced by the severe autoimmune syndrome that results in mice which lack CD4+CD25+ regulatory T cells (86). However, Tregs are also thought to play a role in tumour immunity by suppressing anti tumour responses. This concept is supported by the isolation of tumour specific CD4+ regulatory T cells from tumour infiltrating lymphocytes, as well as the observation that removal of CD4+/CD25+ regulatory cells resulted in the in vivo eradication of tumours in a murine model(87;88).

1.6 Antigen specific immunotherapy of cancer

1.6.1 Vaccination

This is a form of active immunity whereby antigen is administered in order to generate a protective or therapeutic immune response within the host. In tumour immunotherapy, the aim of vaccination is to expand a population of autologous

tumour specific cytotoxic T cells (CTL) in vivo which can mediate tumour regression. Vaccination has seen widespread success in preventing the spread of infectious diseases, and is an attractive concept for tumour immunotherapy as vaccines are cheap, easy to administer on an out-patient basis and have relatively non-toxic side effects. Effective vaccines need to be immunogenic and stimulate T cell and antibody responses, but in the case of cancer vaccines also need to show a measurable clinical response.

Although cancer vaccines based on synthetic peptides, DNA, tumour cells, dendritic cells and several viruses have all been tested, response rates overall have generally been disappointing: a recent review by Rosenberg *et al* collated the data from trials amassing 440 patients (the majority of whom had melanoma) treated with a range of different cancer vaccines between 1995 and 2004. Objective clinical responses were measured according to the Response Evaluation Criteria in Solid Tumours (RECIST) criteria, or defined as at least a 50% reduction in the sum of the diameters of all lesions without any other lesion growing by more than 25% or any new lesions appearing. Using these combined criteria, they saw an overall response rate of only 2.6% (89).

One reason for this may be because vaccination relies on the patients' own immune system to mount a response, and patients may be immunocompromised due to previous chemotherapy, radiotherapy or metastatic disease. It could also be that high avidity CTL are either not present or not expanded as a result of central or peripheral tolerance mechanisms.

As with vaccination against infectious agents, it seems likely that vaccination against tumour antigens will prove most effective when used prophylactically, rather than when a tumour has already arisen. Prophylactic vaccination has been shown to protect against later tumour challenge in murine models (90-93), but active vaccination with TAAs once the animal has an established tumour has not seen the same success. Prophylactic vaccination for tumours caused by chronic viral infection is well established: hepatitis B vaccination has reduced the incidence of hepatocellular carcinoma and a license has recently been granted for a vaccine which targets epitopes of Human Papilloma Virus (HPV) and protects against 70% of cervical cancer (94;95). It is of note that there are no tolerance issues when targeting viral tumour antigens.

1.6.2 Adoptive T cell transfer

In this method, the antigen specific effectors of immunity are directly administered, bypassing the obstacles in the host that might prevent the generation of an effective response in vivo. Large numbers of cells may be expanded in vitro, and can be selected to be of high avidity and tested for anti tumour activity before transfer into patients.

The adoptive transfer of antigen specific T cells was first used to good effect in the treatment of Epstein Barr virus (EBV) and cytomegalovirus (CMV) infection or reactivation occurring in immunosuppressed bone marrow transplant patients . EBV related post transplant lymphoproliferative disease (PTLD) is a significant cause of morbidity and mortality in transplant recipients. EBV-specific T-cell lines, prepared from peripheral blood collected from donors on the day of marrow harvest, have been used both prophylactically (in patients thought to be at risk of developing PTLD), and therapeutically with good clinical effect (96;97). Cytomegalovirus (CMV) infection or reactivation is another cause of morbidity and mortality post bone marrow transplant. Clinical protocols have been developed which isolate CMV-specific T cells from the transplant donor and adoptively transfer them to the patient after transplantation. This has proven effective in the prevention of reactivation and treatment of CMV infection that is unresponsive to antiviral therapy (98-100).

1.6.3 Autologous lymphocyte transfer

Researchers have looked at ways of directing patients' own T cells against tumours. It has previously been shown that mice with established tumours expressing the melanoma-melanocyte differentiation antigen gp100 demonstrated long term tumour regression after receiving gp100 specific CD8 T cells, combined with vaccination and IL-2 (101). In melanoma patients it has been possible to isolate T cells specific for melanoma antigens (e.g. gp100 and MART-1) from extracted tumours: tumour infiltrating lymphocytes (TIL). These cells can be cultured ex vivo to expand their numbers and can then be tested for anti-tumour activity in vitro before adoptively transferring these cells back to patients.

In a study of 13 HLA A2 melanoma patients with progressive disease refractory to standard therapy, TIL which recognised melanoma antigens were isolated from patients and expanded ex vivo. T cells which were reactive against either an A2 positive melanoma cell line or an autologous melanoma cell line in vitro were then selected for adoptive transfer. Patients underwent non-myeloablative chemotherapy

conditioning before receiving on average 7.8 $x10^{x10}$ cells and 9 doses of IL-2. 6 out of 13 patients showed objective clinical responses, with 4 further patients showing mixed responses. This study was extended to include a total of 35 patients, for whom the objective response rate (defined as >50% reduction in tumour size) was 51%, including four patients who showed a complete response. Analysis of 25 of these patients showed a statistically significant correlation between the persistence of antigen specific T cells and objective tumour regression (102).

The significant advantages of this method are the high response rate and the ability to test the T cells before returning them to the patient. However, there are a number of practical limitations with this type of adoptive therapy. It is not always possible to isolate TIL from all patients, nor all tumour types, and it is also a very labour intensive process requiring the expansion of a specific set of cells for each individual patient. Despite these limitations, the proof of principle remains: autologous cells which recognise TAAs can be adoptively transferred into melanoma patients, and these cells can expand and show anti-tumour activity in vivo.

1.7 Targeting WT1 for cancer immunotherapy

WT1 is a TAA which is currently being evaluated by several research groups as a target for the immunotherapy of leukaemia and solid tumours. WT1 encodes a transcription factor which can act as a transcriptional activator or repressor. The carboxyl terminus contains 4 zinc finger domains which mediate DNA binding, whereas the amino terminus consists of a transcriptional repression domain followed by a trans-activation domain (103;104).

WT1 exists as a number of different splice variants and isoforms, the exact function of which have not all been elucidated. Alternative splicing of the WT1 transcript gives rise to four major splice variants which are conserved across mammalian species, each with different functional properties. One splice results in the insertion of the amino acids KTS between zinc fingers 3 and 4, which alters the DNA binding ability of WT1. Another splice results in the insertion of a 17 amino acid sequence encoded by exon 5. There are 24 different isoforms resulting from alternative splicing, different translational start sites, RNA editing or alternative promoters, and a truncated form of WT1 has been described in association with a number of leukaemias and solid tumours (105-109).

Elevated expression of WT1 is a feature of many leukaemias and solid tumours, and high levels of WT1 expression are associated with a poor prognosis in breast cancer and leukaemia (110;111). Thus, a strategy which targets WT1 can be used against a wide variety of tumours and in a large number of patients. In addition, WT1 appears to be important in maintaining the malignant phenotype. When cell lines over-expressing WT1 were treated with WT1 antisense oligomers, the growth of these cells was significantly inhibited in association with a reduction in WT1 protein levels. This indicated that WT1 is essential for the growth of malignant cells and performs an oncogenic rather than a tumour-suppressor gene function in these circumstances (112-114). Tumours often try to evade the immune system by downregulating expression of target antigens. However, if WT1 is required for maintenance of the malignant phenotype, then downregulation of WT1 in response to immunotherapy will adversely affect tumour survival.

Although expression of WT1 in the adult is largely limited to renal podocytes and haematopoetic stem cells, WT1 is required for fetal urogenital development, and expression of WT1 during fetal development is extensive. WT1 is also expressed at low levels in the thymus (115). This is likely to result in central tolerance towards WT1 and the deletion of CTL which recognise WT1 with high avidity, which is a potential problem for a WT1 based immunotherapy approach.

However, WT1 specific T cells can escape central tolerance. It has been demonstrated that vaccination with WT1 peptides in mice and in humans can generate WT1 CTL responses, indicating that tolerance to WT1 is incomplete (93;116;117). Whether vaccine induced WT1 specific T cells are of sufficient avidity to kill WT1 expressing tumour cells in vivo is not clear at present.

1.8 TCR gene transfer

In recent years a new strategy has evolved to facilitate the adoptive transfer of autologous T cells which are specific for TAAs. This technique enables the production of autologous, antigen specific T cells. It overcomes the problem of central tolerance to TAA and the need for the host to mount an immune response against tumour antigens. TCR gene transfer also avoids the requirement for individually tailored therapy which is a drawback seen with the transfer of autologous TIL. Further advantages of this strategy are that the introduced specificity can be targeted against poorly immunogenic targets and the introduced genes can be selected to code for a high affinity TCR.

1.8.1 Structure of the TCR

The αβ TCR is a heterodimer linked by a disulphide bond in the hinge region, which is located above the transmembrane region of each chain. The variable region of the TCR determines its antigen binding specificity. TCR diversity is generated by TCR gene rearrangement in the thymus, and it is in complementarity determining region (CDR) 3 that diversity is concentrated. Each TCR variable region contains three CDRs which interact with the peptide:MHC complex. CDRs 1 and 2 are mainly in contact with the MHC molecule, whereas the CDR3 region interacts extensively with peptide residues. Each T cell expresses a single TCR specificity. (118;119).

1.8.2 TCR:CD3 complex

The CD3 complex is required in order for a TCR to be expressed at the cell surface, and to transduce signals intracellularly upon antigen binding by the TCR. The CD3 complex is composed of 4 invariant chains: gamma, delta, epsilon and zeta. One epsilon and one delta chain form a heterodimer, as do one gamma and one epsilon chain. Whereas these heterodimers are largely extracellular and are required for TCR surface expression, two zeta chains form a homodimer which is largely intracellular (Figure 1.1). The CD3 gamma, delta and epsilon proteins are encoded on chromosome 11, separately from the zeta chain on chromosome 1. Expression of the CD3 genes is restricted to cells of T lymphocyte lineage. The CD3 complex is expressed early in thymocyte development and precedes TCR gene rearrangement and expression: CD3 epsilon, delta and gamma mRNA can be isolated from the earliest thymocytes (120;121). The TCR/CD3 complex is assembled in the ER and the zeta chains are the last component to be integrated before the complex is transport to the cell surface. (122-124).

1.8.3 TCR signal transduction

Binding of a TCR to its cognate peptide:MHC complex results in signal transduction which ultimately leads to changes in gene transcription, giving rise to a range of T cell effector functions. Clustering of TCRs upon antigen binding leads to rearrangement of the cytoskeleton and the formation of a supramolecular adhesion complex (SMAC), in which the TCRs co-localise with the necessary coreceptors (such as CD4 and CD8), other signalling molecules and adhesion molecules. Formation of a SMAC on a T cell then causes the TCR ligands on an APC or target cell to be concentrated in proximity with the SMAC. The site of contact between the T

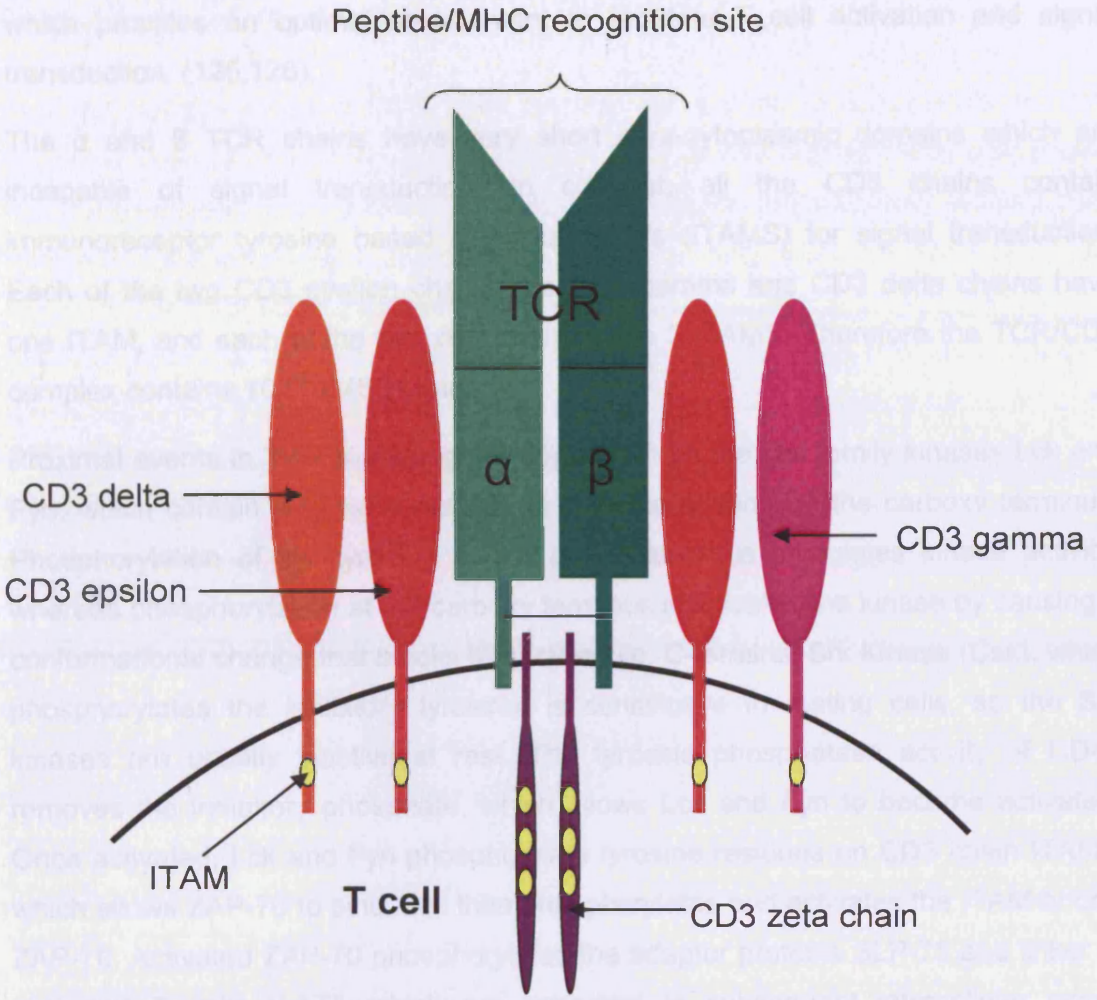

Figure 1.1: the TCR:CD3 complex. The αβTCR associates with a complex of invariant CD3 chains, which are necessary for both TCR expression on the cell surface as well as signal transduction upon antigen recognition. Two CD3ε chains form heterodimers with a CD3γ and a CD3δ chain, respectively, and two CD3 ζ chains form a homodimer. The intracellular regions of the CD3 chains contain immunoreceptor tyrosine-based activation molecules (ITAMs). Antigen binding to the TCR results in phosphorylation of the ITAMS, initiating signal transduction pathways which ultimately lead to changes in gene transcription and a range of T cell effector functions.

cell and its target is called the immunological synapse: a highly organised interface which provides an optimal environment to facilitate T cell activation and signal transduction. (125;126).

The α and β TCR chains have very short intra-cytoplasmic domains which are incapable of signal transduction. In contrast, all the CD3 chains contain immunoreceptor tyrosine based activation motifs (ITAMS) for signal transduction. Each of the two CD3 epsilon chains, the CD3 gamma and CD3 delta chains have one ITAM, and each of the two zeta chains have 3 ITAMS. Therefore the TCR/CD3 complex contains 10 ITAMS in total.

Proximal events in TCR signalling are dependent on the Src family kinases Lck and Fyn, which contain a tyrosine residue at both the amino and the carboxy terminus. Phosphorylation of the tyrosine at the amino terminus stimulates kinase activity, whereas phosphorylation at the carboxy terminus inactivates the kinase by causing a conformational change that blocks the active site. C-terminal Srk Kinase (Csk), which phosphorylates the inhibitory tyrosine, is constitutive in resting cells, so the Src kinases are usually inactive at rest. The tyrosine phosphatase activity of CD45 removes the inhibitory phosphate, which allows Lck and Fyn to become activated. Once activated, Lck and Fyn phosphorylate tyrosine residues on CD3 chain ITAMs, which allows ZAP-70 to bind. Lck then phosphorylates and activates the ITAM-bound ZAP-70. Activated ZAP-70 phosphorylates the adaptor proteins SLP-76 and linker of activated T cells (LAT), which are important in subsequent intracellular signal transduction (127-129).

Phosphorylation of the zeta chain ITAMs is an important early event in TCR signal transduction. The importance of the zeta chain is illustrated when zeta chain expression is downregulated in association with a number of chronic infections and malignancies. In these circumstances, T cells with reduced zeta expression are still able to express TCR/CD3 complexes on the cell surface, but the T cells are functionally impaired as a result of impaired signal transduction (130).

1.8.4 Retrovirally mediated TCR gene transfer

TCR gene transfer makes it possible to target T cells to antigens of interest, including tumour antigens. The allo-restricted strategy can be used to isolate high avidity CTL which are specific for the target antigen (131-133). The TCR α and β chains can then be isolated from these CTL and cloned into vectors, which are then used to transduce patients' T cells. The recipient T cell expresses the introduced TCR on its

surface, hence its specificity has been redirected, as it will now recognise the tumour antigen of interest (Figure 1.2).

The first demonstration of TCR gene transfer being used to successfully transfer TCR into target (hybridoma) cells occurred in 1986, at which point it was also shown that the expressed TCR retained the MHC-restricted antigen specificity of the T cells from which the genes were originally cloned (134). Since then, this technology has been used to redirect the specificity of T cells against a range of viral, tumour and other targets. Clay *et al* first demonstrated that TCR gene transfer could be used to generate human T cells which released interferon gamma in response to tumour cells (135). Furthermore, by transferring allo-restricted TCRs into autologous T cells, it is possible to circumvent tolerance and generate high avidity autologous cells which recognise TAA (136).

Since commencing my PhD, TCR gene transfer has seen clinical success in a recent Phase I trial in melanoma patients. Retroviral gene transfer was used to transduce peripheral blood lymphocytes, taken from patients with melanoma, with the genes encoding the α and β chains of the anti MART-1 TCR. The genes for this TCR, which recognises a melanoma associated antigen, were cloned from a TIL clone obtained from a melanoma patient who showed a good clinical response to adoptive T cell therapy (137). This study, while small, demonstrated proof of principle: retroviral TCR gene transfer can be used to confer anti tumour specificity upon a large number of T cells, and that these T cells can engraft in cancer patients and persist at high levels long term. However, only 2/17 patients responded clinically to treatment with genetically modified T cells (12%), suggesting that further improvements are needed to enhance the therapeutic efficacy of TCR modified cells.

1.8.5 Augmenting the TCR transfer strategy

A number of strategies are being investigated in order to augment the TCR gene transfer concept. The majority of these involve manipulation of the TCR itself, such as making alterations to the TCR constant region in order to reduce mispairing and increase expression.

1.8.6 Competition for CD3

One of the aims of this work is to investigate whether the sensitivity of a TCR can be increased *without* altering the TCR itself. The availability of the CD3 complex may be

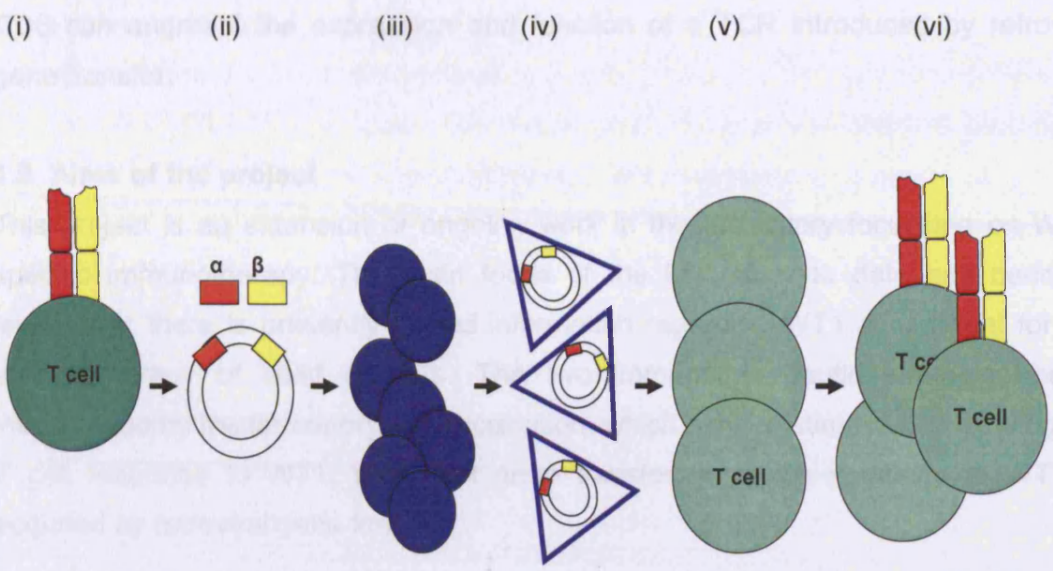

Figure 1.2: TCR gene transfer

(i) A T cell bearing the appropriate TCR is identified, (ii) the α and β TCR chain genes are isolated and cloned into a retroviral vector, (iii) the vector is used to transfect a packaging cell line which produces viral particles containing the genes of interest: (iv). (v) Target T cells are transduced with the recombinant viral particles; (vi) when the genes integrate into the host DNA the target cells express the desired TCR

a rate limiting step for the expression of TCRs, especially following TCR gene transfer, when there are both endogenous and introduced TCRs competing for surface expression. I have therefore chosen to investigate whether co-transduction of CD3 can augment the expression and function of a TCR introduced by retroviral gene transfer.

1.9 Aims of the project

This project is an extension of ongoing work in the laboratory focussing on WT1-specific immunotherapy. The main focus of the laboratory to date has been on leukaemia; there is presently limited information regarding WT1 as a target for the immunotherapy of solid cancers. The two immunotherapeutic strategies being investigated by the laboratory are vaccination, which aims to stimulate an autologous T cell response to WT1, and TCR gene transfer, in which immunity to WT1 is acquired by retroviral gene transfer.

The first aim of this project is to evaluate WT1 as a target for the immunotherapy of prostate cancer, and explore autologous T cell responses to WT1 in patients with prostate cancer.

The second aim of this project is to enhance the efficacy of TCR gene transfer. Firstly I will assess whether CD3 is a rate limiting step for TCR expression in T cells, and secondly I will investigate whether the expression and sensitivity of a TCR introduced by retroviral gene transfer can be increased by the co-transduction of CD3.

1.9.1 Hypotheses

In this thesis I will test the following working hypotheses:

1. WT1 is aberrantly expressed in prostate cancer
2. WT1 specific CTL are detectable in patients with prostate cancer
3. Impaired T cell function in prostate cancer patients can be reversed with IL-15
4. Co-transfer of TCR and CD3 can enhance the TCR expression of gene modified cells
5. Co-transfer of TCR and CD3 can enhance the antigen specific function and sensitivity of gene modified cells

Chapter 2: Materials and Methods

2.1 *In vitro* cell culture

2.1.1 Tumour cell lines and culture condition

Ecotropic Phoenix cells (Phoenix-eco) were used as the packaging line for production of retroviral particles. They were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Cambrex) supplemented with membrane filtered 10% heat-inactivated foetal calf serum (HI FCS) (Biowest, Nuaille, France), 1% penicillin/streptomycin (Invitrogen) and 2mM L-glutamine (Sigma-Aldrich)(Eco medium).

All other tumour cell lines were cultured in Normal Growth Medium (NGM): Roswell Park Memorial Institute 1640 (RPMI) medium (Invitrogen, Paisley, UK), supplemented with 10% HI FCS, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. All cell lines were grown in suspension other than Phoenix-eco cells, which is an adherent cell line. All cells were cultured in a 37°C humidified incubator with 5% CO2.

The T2 cell line is a Transporter Associated with Antigen Processing (TAP) deficient cell line whose HLA-A2 Class 1 molecules are inefficiently loaded with peptide. Class I molecules can be stabilised at the cell surface by loading them with exogenous peptide (138). RMA-S is the murine equivalent: a TAP deficient derivative of the RMA cell line which can efficiently present exogenously loaded peptide and was derived from a Rauscher virus-induced C57/B6N T cell lymphoma (139;140). On the day prior to loading RMA-S cells with peptide they were placed in a waterbath at 26°C overnight, in a sealed tissue culture flask, in gassed NGM at a density of 5-10x10^5/ml. This temperature induction increases the surface expression of MHC Class I molecules so that they can be more efficiently loaded with exogenous peptide (141)

$58^{\alpha-\beta-}$ cells were grown in NGM. This is a TCR negative variant of the D0-11.10 T cell hybridoma cell line (142).

2.2 Patient clinical samples

2.2.1 Ethics committee approval

Ethics committee approval for this research was granted by the Royal Free Hospital and Medical School Local Research Ethics Committee (REC reference number:

05/Q0501/161). All clinical samples were obtained after having first gained written informed consent from the patient.

2.2.2 Preparation of mononuclear cells from whole blood

PBMC were separated from blood by density gradient separation. The blood sample was mixed 1:1 with warm RPMI 1640, then 30ml aliquots were gently layered onto 15mls of Lymphoprep (Fresinius Kabi, Norway) in a 50ml Falcon tube. After centrifugation at 2000rpm for 30 mins (without centrifuge braking), an interface containing PBMC was visible between the plasma above and lymphoprep/red cell pellet below. The interface was pipetted off and re-suspended in RPMI. A further three washing steps were performed at 1600, 1300 and 1100rpm (with the brake on). Cells to be stored were counted, resuspended in membrane filtered freezing medium (90% FCS, 10% Dimethyl Sulfoxide (DMSO), [Sigma, UK]) and aliquoted into cryovials. After 30 minutes on ice, cryovials were stored in -80°C freezer for 24 hours before transfer into liquid nitrogen.

2.3 WT1 Immunohistochemistry

Prostate cancer tissue array PR 951 was obtained from US Biomax Inc. (Ijamsville, US). The immunohistochemistry was carried out by the Department of Histopathology, Hammersmith Hospital who kindly provided the following protocol:

a) slides were stained on a Bond max machine, which automates the de-waxing and antigen retrieval steps as well as the staining.
b) Antigen retrieval in the ER2 solution (pH 9.0) for 30 min
c) The detection system is from Vision Biosystems (BondPolymer Define)
d) Primary antibody (Novocastra) incubation at 1/400 for 20 mins
e) Post primary antibody incubation for 15 min
f) Polymer incubation for 15 min
g) DAB for 10 min, enhance for 5 min

For each immunohistochemical reaction, a negative control without primary antibody was included. All samples were analysed by myself and Dr Fabrizio Corsi from the Department of Histopathology, Hammersmith Hospital. Both researchers were blinded to the clinical characteristics of the samples being analysed.

2.4 Flow cytometry

Unless otherwise stated, all flow cytometric agents were purchased from BD Biosciences, Oxford, UK.

2.4.1 Staining for cell surface molecules

Direct immunofluorescence was used to stain for cell surface molecules as follows: approximately 5 $x10^5$ cells were used per sample. Cells were washed in cold staining buffer (PBS with 1% HI FCS) and resuspended in 50µl staining buffer containing the appropriate dilution of the relevant monoclonal antibody. Samples were incubated at 4°c for 25 minutes in the dark, washed twice with staining buffer and resuspended in 300µl staining buffer prior to analysis. Propidium iodide (PI; Sigma Adrich) was added to give a final concentration of 0.1µg/ml to distinguish dead cells, which take up PI. PI gives a signal in both the FL2 and FL3 channels, allowing PI positive cells to be excluded from further analysis. Flow cytometry antibodies used were: FITC labelled anti-human CD3ε, APC labelled anti-murine TCR β chain constant region, anti-murine Vα2 and anti-human CD8, PE labelled anti-murine CD3ε, anti-murine vβ11, anti-human CD8-PE and anti-human Vβ2.1 (Immunotech)

2.4.2 Tetramers and peptides

2.4.2.1 Synthetic Peptides

All peptides were obtained from Proimmune, Oxford, UK. The synthetic peptide pWT126 corresponds to amino acids 126-134 (RMFPNAPYL) of the Wilms' tumour protein (WT1). The synthetic peptide pMelan-A corresponds to amino acids 26-35 (ELAGIGILTV) of the Melan-A protein, but with a leucine substituted for the alanine at position 27. This amino acid substitution results in an increased binding of the peptide to the HLA-A*0201 molecule and a corresponding increase in antigenicity of the peptide(143;144), but tetramers containing the modified pMelan-A also bind T cells which recognise the unmodified peptide (145).

The influenza virus A NP-derived peptide, pNP366 (ASNENMDAM), and the control peptides pMDM100 and pSV9 all bind to the H2-K^b molecule. The pMDM100 (YAMIYRNL) is derived from the murine double minute (MDM) 2 protein, and pSV9 (FAPGNYPAL) is derived from the Sendai virus.

Peptides were re-constituted from powder to a 20mM stock solution in distilled water (45%), DMSO (50%) and dithiothretol (DTT) (5%). 2mM aliquots were then prepared (to minimise repeated freeze-thawing of stock) and stored at -20°C

2.4.2.2 Tetramer staining

Phycoerythrin (PE)-labelled tetramers consisting of a specific peptide bound to H2-K^b or HLA-A*0201 Class I molecules were used to detect antigen specific CD8 T cells. The influenza tetramer was obtained from Proimmune, Oxford, UK. All other tetramers were obtained from Beckman Coulter, France, and contained an amino acid substitution (alanine replaces valine at position 245) in the α3 chain of the MHC Class I molecule, resulting in a reduced interaction with CD8 molecules. These mutated tetramers have been shown to display reduced non-specific binding resulting from interaction with CD8 molecules, while not reducing specific binding to TCR molecules (146;147).

Tetramer staining on PBMC was performed on between 1-5 $x10^6$ cells. (When low frequency events were expected, a greater number of cells were stained and a greater frequency of events recorded.) Cells were washed in FACS buffer and resuspended in 50µl of a 1 in 50 dilution of tetramer for 20 minutes at room temperature, in the dark. Cells were washed once in FACS buffer before being stained with directly conjugated antibodies to CD3 and CD8. Samples were incubated at 4°C for 20 minutes in the dark, washed twice in FACS buffer and resuspended in 400µl FACS buffer for analysis. PI was added to distinguish dead from viable cells. Analysis was performed on gated viable, CD8 positive T cells.

2.5 T cell culture

Standard T cell culture medium consisted of RPMI 1640 containing 10% HI FCS, 1% penicillin/streptomycin and 1% L-glutamine.

2.5.1 T cell culture day 0

Mononuclear cells or murine splenocytes were grown *in vitro* and stimulated every 7-10 days in a peptide specific manner. Cells were suspended in NGM at a density of $3x10^6$ PBMC in a 24 well plate containing 2ml of media and peptide at a final concentration of 10 µM. The cytokines IL-2 (Roche, Lewes, UK) and IL-7 (R&D Systems, Abingdon, UK) were added to the medium on day 0 at a concentration of 20units/ml and 2.5ng/ml, respectively. Alternatively, IL15 was added to the medium on day 0 at a concentration of 50 units/ml (R&D Systems, Minneapolis). The cells were incubated at 37°C, 5% CO2 for 7-10 days.

2.5.2 Restimulation of T cell cultures

Restimulation was carried out every 7-10 days after tetramer staining had been performed. Restimulation involved plating the cells at a density of 5 x 10^5 per 2 ml of T cell medium containing IL2 at a concentration of 20 units/ml, or IL15 at a concentration of 50 units/ml. Cultures were stimulated using irradiated (80 Gy) T2 or RMA-S cells (for human or murine T cells, respectively,) loaded with 100 µM of the relevant peptide (2x10^5 T2 or RMAS cells per well). Irradiated (30 Gy) PBMC from healthy HLA-A2 positive donors obtained from the National Blood Service (Colindale, UK), or C57BL/6 splenocytes, were used as feeder cells (2x10^6 cells per well)

2.6 Magnetic bead sorting

2.6.1 Depletion of endogenous Vβ11 positive splenocytes

When Vβ11 depleted splenocytes were required for transduction, splenocytes were separated prior to activation, on day 2 of the transduction protocol, using MACS PE beads according to the manufacturer's protocol (Miltenyi Biotec, Bisley, Surrey, UK). Briefly, cells were surface stained with Vβ11-PE antibody for 30 minutes on ice, before being washed in 1ml MACS buffer (PBS pH7.2 containing 0.5% BSA and 2mM EDTA) per 1 x10^7 cells and centrifuged for 10 minutes at 300xg. Cells were then incubated with 40µl PE microbeads and 60µl MACS buffer per 1x10^7 cells, on ice, for 20 minutes. Cells were then washed with 1ml MACS buffer per 1 x10^7 cells and centrifuged for 10 minutes at 300xg.The pellet was then resuspended in 500µl MACS buffer per 1 x10^8 cells. Magnetic separation was performed using an MS column placed in the magnetic field of a MiniMACS separator. The column was prepared for use by applying 500µl MACS buffer and allowing it to run through. The cell suspension was then applied to the column, which was subsequently washed three times with 500µl MACS buffer, collecting the unlabelled, Vβ11 depleted cell fraction. Cells were then counted and activated with ConA and IL-7 as previously described.

2.7 Generation of TCR transduced T cells

2.7.1 Retroviral vectors

The retroviral vectors pMX-TCRα-IRES-TCRβ (F5 TCR) and pCL-Eco were a kind gift from T. Schumacher (Nederlands Kanker Institute, Amsterdam) (148). pCL-Eco is a retroviral vector encoding an ecotropic envelope, which is used to enhance retroviral transduction of murine cells. The pMX vector is based on Moloney murine leukaemia virus. The F5 TCR recognizes the influenza virus A NP (NP366–379) peptide in the context of murine D^b MHC class I. The retroviral vector pMSCV-CD3δ-

CD3γ-CD3ε-CD3ζ-IRES-GFP (CD3δ-GFP) is a murine stem cell virus based vector and was a kind gift from D.Vignali (University of Tennessee Medical Center, USA) (149).The retroviral vector pMP71-TCRα-2A-TCRβ (WT1 TCR) is a myeloproliferative sarcoma virus based vector. The WT1 TCR recognises the WT126 peptide in the context of HLA-A*0201. The constant regions of the α and β TCR chains have been replaced with murine constant regions, which also contain additional cysteine residues and have been codon optimized, as described previously (150;151). The retroviral vector pMP71-CD3ζ-CD3ε-CD3γ-CD3δ-IRES-GFP (CD3ζ-GFP), in which the order of the CD3 chains is reversed, was constructed by Geneart (Regensburg, Germany). The 2A sequences linking the CD3 chains are unchanged from the CD3δ-GFP vector, but the CD3 genes have been codon optimised and cloned into the pMP71 vector. In the retroviral vector pMP71-rWT1-IRES-GFP (GFP control), the WT1 gene has been inserted into the cloning site in reverse orientation such that only the GFP is translated from this vector.

2.7.2 Transfection and generation of retroviral particles

$1.8x10^6$ Phoenix-eco cells were seeded in a $60cm^2$ (96x21mm) sterile tissue culture treated Petri dish (TTP, Trasadingen, Switzerland) in 10ml of eco medium 24 hours prior to transfection and incubated in a humidified atmosphere at 37°C with 5% CO2 (day 0). Transfection took place on day 1. A transfection mixture was prepared containing the following: 7.5µl (7.5µg) pCL-eco, 18µl 2M Calcium Chloride (CaCl2)(Invitrogen), either 10 µl (10µg) of TCR expressing vector *or* 5 µl (5µg) of CD3 expressing vector *or* 5 µl (5µg) of GFP expressing vector, plus dH20 to make a total volume of 150 µl. Control experiments were carried out in all experiments, with the vector being replaced with an equivalent volume of dH20. The transfection mixture was then added drop-wise to 150µl of 2xHBS (Invitrogen) while air was being bubbled through it to form a fine precipitate. The transfection mixture was then left for 30 minutes at room temperature while the medium was removed from the Phoenix eco cells to be replaced with 5mls of fresh medium, taking care not to disrupt the cells. After the 30 minute incubation the DNA transfection mix was added drop wise to the Phoenix cells, which were then incubated in a humidified atmosphere at 37°C with 5% CO2. 18 hours post transfection (day 2), the medium was removed, the Phoenix cells carefully washed once with warm PBS and 5ml of NGM was placed on the Phoenix cells which were returned to the incubator for a further 24 hours.

2.7.3 Retroviral transduction

Freshly isolated C57BL/6 (H2b) splenocytes were activated for 2 days prior to transduction with 2µg/ml concavalin A (conA) (Sigma-Aldrich) and 1ng/ml recombinant IL-7 (R&D Systems) (day 1). On day 3, 6-well non-tissue culture treated plates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) were coated with 2.5ml 30 µg/ml Retronectin (Takara-Bio Inc, Otsu, Japan) per well and incubated at room temperature for 2-4 hours. Retronectin is a human fibronectin peptide which increases transduction efficiency by co-localising viral particles and target cells on the retronectin molecules (152). Following this incubation the Retronectin was removed and the plates were blocked with 3ml/well 2% BSA (Sigma-Aldrich) diluted in PBS for 30 minutes at room temperature. This was then removed and the plates washed twice with PBS. $2.5x10^6$ $58^{\alpha-\beta-}$ cells or activated splenocytes were added to each well. Viral supernatant was harvested and centrifuged before 2ml of supernatant was added to the cells along with NGM to make a total volume of 5ml per well. For example: cells transduced with the CD3 vector would receive 2ml supernatant and 3ml NGM, whereas cells transduced with the CD3 vector and the F5 TCR vector would receive 2ml of CD3 supernatant, 2ml of F5 supernatant and 1ml of NGM. In addition, splenocyte cultures received 100U/ml recombinant human IL-2 (Chiron). On days 4 and 6, cells were harvested and resuspended in 6ml fresh NGM (containing 100U/ml for splenocyte cultures) in a tissue culture treated 6 well plate. Transduction efficiency was assessed by flow cytometry on day 6.

2.7.4 Assessment of transduction efficiency by flow cytometry

Transduction efficiency was determined by staining with $5\text{-}10x10^5$ cells with tetramers and surface antibodies in FACS buffer as described above. Samples were then resuspended in FACS buffer and analysed using an LSRII flow cytometer and FACSDiva software

2.8 In vitro functional assays

2.8.1 IL-2 ELISA

On day 5 after transduction $5x10^4$ peptide loaded RMA-S or T2 cells were incubated with $5x10^4$ transduced splenocytes or $1x10^5$ transduced $58^{\alpha-\beta-}$ cells, in 200ml NGM, in triplicate. After 24h, the supernatant was harvested and tested in an IL-2 ELISA assay using a Beckon Dickinson OptEIA™ mouse ELISA set according to the manufacturer's instructions (BD Biosciences). Briefly, 96-well ELISA plates (Nunc) were coated with 100µl per well with anti-mouse IL-2 capture antibody and incubated overnight at 4°C. Plates were washed three times and blocked for 1 hour at RT with

200µl assay diluent. Plates were then washed three times. Recombinant mouse IL-2 dilutions were prepared in assay diluent as standards. Standards were prepared in triplicate from 200pg/ml, in double dilutions to 3.1pg/ml. Supernatant was harvested from stimulation plates after 24 hours. 100µl standard/supernant was added to each well and incubated at RT for 2 hours. Plates were washed 5 times and 100µl of working detector (biotinylated anti-mouse IL-2 and 1:500 avidin-horseradish peroxidise conjugate) added to each well. Plates were incubated at RT for 1 hour, then washed 7 times. 100µl of substrate solution was added to each well and incubated for 30 minutes in the dark, after which 50µl stop solution was added. The absorbance at OD450 was read using an ELISA plate reader (Titertek). Data are presented as the mean IL-2 concentration (pg/ml) +/- standard deviation of triplicate values.

2.8.2 IFN-γ ELISA

IFN-γ was measured in the culture supernatant of stimulated transduced $58^{\alpha-\beta-}$ cells or T cells using a Becton Dickinson OptEIA™ mouse IFN-γ ELISA kit (BD Biosciences) according to the manufacturer's protocol (as described above). Data are presented as the mean IFN-γ concentration (pg/ml) +/- standard deviation of triplicate values.

2.8.3 Intracellular cytokine staining

Intracellular IFNγ detection was performed using a BD Cytofix/Cytoperm™ Fixation/Permeabilisation Kit according to the manufacturer's instructions. 1 x 10^6 bulk transduced T cells stimulated with 5 x 10^5 T2 cells loaded with 10 µmol/L of either pWT126 or pMelan-A peptide. After 2 hours, Brefeldin A (Sigma) was added at a concentration of 10 µg/mL to block cytokine secretion. After another 12 hours, cells were stained with anti-murine CD8 APC and incubated at 4°C for 20 minutes. Following this, cells were resuspended in Fixation/Permeabilization solution for 20 min at 4°C. After another two washes, cells were stained with IFNγ-PE for 30 min at 4°C in the dark. After a further 2 washes, samples were resuspended in 300 µL of PBS/1% FCS prior to flow cytometric analysis.

2.8.4 IFN-γ ELISPOT

This assay was performed performed using an IFNγ ELISPOT kit (BD Biosciences) according to the manufacturer's instructions. Autologous PBMC (2×10^5/well) were added together with the indicated number of responder T cells (2×10^5/well) and peptide (5µg/ml) in 200µl NGM and incubated for 20 h at 37°C, in triplicate. Cells

were then discarded, plates were washed and developed with a second antibody to human IFNγ (biotinylated) and streptavidin-alkaline phosphatase according to the manufacturer's instructions. Responder cells and stimulators loaded with an irrelevant peptide, and responder cells without stimulator cells were used as negative controls. 200 ng/ml of staphylococcal enterotoxin B (SEB) was used as a positive control on $5x10^4$ cells. Results are expressed as the mean number of spot forming units (SFU) per 10^6 cells from triplicate wells. Only cell samples with > 80% viability were analyzed, and only assays with <50 SFU/10^6 cells for the negative control and >500 SFU/10^6 cells after SEB stimulation were considered valid.

2.9 Annexin V staining

Annexin V staining was performed according to the manufacturer's instructions (BD Biosciences). Cells were washed twice in cold PBS and resuspended in 1x binding buffer at a concentration of $Ix10^6$ cells/ml. 100µl of the solution was transferred to a 5ml culture tube before adding 5µl of Annexin V-APC and 5µl of PI. The cells were gently vortexed and incubated for 15 min at 25°C in the dark. 400µl of binding buffer was then added and cells were analysed by flow cytometry within 1 hour.

2.10 Animals

Normal C57BL/6 (H2b) female mice were used as tissue donors. All animals were housed in a specific pathogen-free environment in accordance with institutional guidelines for animal care. C56BL/6 mice were obtained from Harlan, Oxon, UK.

Chapter 3: Evaluating WT1 as a target for the immunotherapy of prostate cancer

3.1 Introduction

Prostate cancer is the commonest cancer in men in this country, with over 35,000 new diagnoses each year, and the incidence worldwide is increasing steadily (153). The three most common treatment modalities, surgery, radiotherapy and androgen ablation, are all associated with significant side effects and reduced quality of life (154). It is therefore desirable to develop new immunotherapy options with the potential to selectively eliminate prostate cancer cells. Cytotoxic T cells (CTL) are important effectors of anti-tumour immune responses (155). The vast majority of CTL-recognised targets described to date are tumour-associated antigens such as the Wilms' tumour antigen (WT1), which is currently being evaluated for the immunotherapy of a number of leukaemias and solid tumours. However, there is currently limited information regarding the expression and immunogenicity of WT1 in prostate cancer.

Immunological tolerance to tumour associated antigens such as WT1 remains a potential barrier to successful T cell immunotherapy. T cell unresponsiveness in the presence of tumours has been described in melanoma patients and in murine tumour models (12;156;157). Although interleukin 2 (IL2) was shown to reverse unresponsivenessis in classical T cell anergy, recent experiments in transgenic mice revealed that T cell tolerance was readily reversed by interleukin 15 (IL15), but not IL2 (158;159).

The aim of this chapter is to evaluate WT1 as a target for the immunotherapy of prostate cancer. I assessed WT1 expression in prostate cancer samples and evaluated whether there are measurable WT1 CTL in prostate cancer patients. Furthermore, I investigated whether these CTL could be expanded *in vitro* by peptide specific stimulation in the presence of different cytokines, and whether they could produce IFNγ in response to antigen specific stimulation. This information would inform the design of immunotherapeutic strategies for prostate cancer patients.

3.2 Results

3.2.1 WT1 expression in prostate cancer and normal controls

I assessed WT1 protein expression by WT1 immunohistochemistry. Prostate tissue microarray PR 951 was obtained from US biomax; patient characteristics can be found in Table 3.1. Immunohistochemistry was performed on fixed prostate cancer tissue using an IgG1 murine monoclonal antibody directed against amino acids 1-181 of the N-terminal of the human WT1 protein. The conditions used to monitor WT1 protein expression were those currently used in the diagnostic service laboratory in the Department of Cytology at the Hammersmith Hospital, London, where WT1 expression is routinely monitored in patient samples. This assay was previously validated using kidney and ovarian carcinoma tissue as a positive control for WT1 protein expression, and skeletal muscle as a negative control. As a further negative control for non-specific binding by the secondary antibody, the primary antibody was replaced with assay diluent. The tissue arrays were graded by myself and Dr Fabrizio Corsi of the Department of Cytology, Hammersmith Hospital; both researchers were blinded to the patient characteristics.

Tissue array PR 951 contained duplicated cores of 36 prostate adenocarcinomas and 8 normal prostate samples. Reproducibility between the duplicates was high: there was discordance for only 1 duplicate sample, where one core showed cytoplasmic staining only, and one showed cytoplasmic as well as nuclear staining. (This sample was counted as showing nuclear as well as cytoplasmic staining in the final analysis.) Of the normal prostate samples, 100% displayed cytoplasmic staining only for WT1, with 0% nuclear staining (Figure 3.1, a). Of the adenocarcinoma samples, 56% showed cytoplasmic staining only, 39% stained in the cytoplasm as well as the nucleus (Figure 3.1, b) and 5% did not stain at all with the WT1 antibody. Where nuclear staining was present, it was heterogenous and the proportion of nuclei which were WT1 positive ranged from 40-60%. The difference in nuclear WT1 staining between normal and adenocarcinoma samples was statistically significant to $p<0.036$ using Fisher's Exact test. The degree of differentiation of the tumours seen histologically (Gleason grade) was not significantly associated with nuclear WT1 staining. 14 out of 29 T3/T4 cancers (which extend through the prostate capsule) showed nuclear WT1 expression, whilst all seven T2 cancers (enclosed within the capsure) in this series were negative for nuclear WT1 expression, although this was not a statistically significant difference.

Table 3.1 Characteristics of 36 cases of localised prostate cancer with 8 matched normal prostate tissue samples, from tissue array PR951, on which WT1 immunohistochemistry was performed.

The Gleason score refers to the degree of differentiation of the tumours seen histologically, with 5 representing poorly differentiated tumours. Each sample receives two scores from 1-5 (e.g.: 3+4). Staining is graded from zero to +++.

ID	Age	Diagnosis	TNM	Gleason	Nuclear	Cytoplasmic
1	44	Malignant adenocarcinoma	T3N0M0	7	+	+
1	44	Normal prostate matched tissue	-	-	-	+
2	63	Malignant adenocarcinoma	T4N0M0	10	+	+
2	63	Normal prostate matched tissue	-	-	-	+
3	70	Malignant adenocarcinoma	T2NOMO	7	-	+
3	70	Normal prostate matched tissue	-	-	-	+
4	70	Malignant adenocarcinoma	T3N0M0	7	+	+
4	70	Normal prostate matched tissue	-	-	-	+
5	69	Malignant adenocarcinoma	T3N0M0	7	-	+
5	69	Normal prostate matched tissue	-	-	-	+
6	65	Malignant adenocarcinoma	T3N0M0	9	-	+
6	65	Normal prostate matched tissue	-	-	-	+
7	62	Malignant adenocarcinoma	T2N0M0	7	-	+
7	62	Normal prostate	-	-	-	+

		matched tissue				
8	69	Malignant adenocarcinoma	T2N0M0	7	-	+
8	69	Normal prostate matched tissue	-	-	-	+
9	63	Malignant adenocarcinoma	T3N0M0	8	-	+
10	53	Malignant adenocarcinoma	T4N0M0	9	+	+
11	63	Malignant adenocarcinoma	T4N0M0	9	+	+
12	62	Malignant adenocarcinoma	T4N0M0	9	-	+
13	75	Malignant adenocarcinoma	T3N0M0	9	+	+
14	70	Malignant adenocarcinoma	T3N0M0	8	-	+
15	72	Malignant adenocarcinoma	T4N0M0	8	-	+
16	57	Malignant adenocarcinoma	T4N0M0	7	+	+
17	68	Malignant adenocarcinoma	T3N0M0	8	+	+
18	66	Malignant adenocarcinoma	T3N0M0	8	+	+
19	72	Malignant adenocarcinoma	T3N0M0	9	-	+
20	63	Malignant adenocarcinoma	T3N0M0	9	+	+
21	59	Malignant adenocarcinoma	T2N0M0	6	-	+
22	70	Malignant adenocarcinoma	T3N0M0	7	-	+
23	71	Malignant adenocarcinoma	T2N0M0	7	-	+
24	58	Malignant	T3N0M0	9	-	+

		adenocarcinoma				
25	69	Malignant adenocarcinoma	T3N0M0	7	-	+
26	67	Malignant adenocarcinoma	T3N1M0	9	-	+
27	70	Malignant adenocarcinoma	T4N0M0	7	-	+
28	60	Malignant adenocarcinoma	T3N0M0	9	-	+
29	66	Malignant adenocarcinoma	T3N0M0	9	+	+
30	73	Malignant adenocarcinoma	T2N0M0	7	-	-
31	65	Malignant adenocarcinoma	T4N0M0	8	+	+
32	59	Malignant adenocarcinoma	T3N0M0	9	-	+
33	64	Malignant adenocarcinoma	T3N0M0	10	+	+
34	71	Malignant adenocarcinoma	T3N0M0	9	-	-
35	64	Malignant adenocarcinoma	T2N0M0	7	-	+
36	68	Malignant adenocarcinoma	T3N0M0	9	+	+

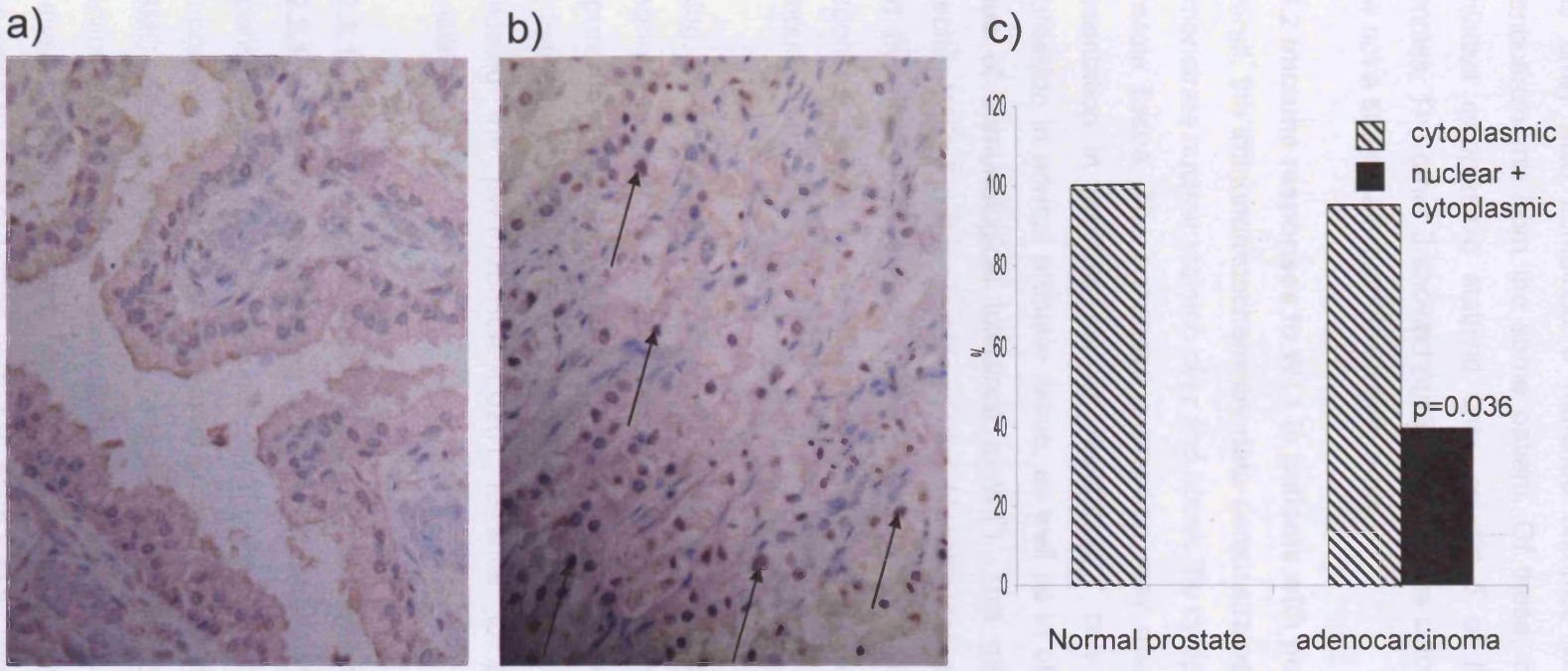

Figure 3.1: Immunohistochemical detection of WT1 in (a) normal prostate tissue and (b) prostate adenocarcinoma tissue.

A murine monoclonal antibody directed against amino acids 1-181 of the N terminal of the human WT1 protein was used on a tissue array comprising duplicates of 36 cases of prostate cancer and 8 normal prostate tissue samples. Positive nuclear WT1 immunoreactivity is visualised as brown (arrows). Nuclei were counterstained with hematoxylin (blue: negative). Original magnification: x400. c: Bar graph showing the frequency with which prostate tissue samples showed cytoplasmic staining or additional nuclear staining.

This array also contained 8 matched samples of normal prostate tissue and adenocarcinoma from the same patient. Of these, all 8 normal prostate samples exhibited cytoplasmic staining only, as did 5 of the matched adenocarcinoma samples. The other 3 showed nuclear as well as cytoplasmic staining, although this was not a statistically significant difference.

3.2.2 Immune responses to WT1 in patients with prostate cancer

Overall, the immunohistochemistry data demonstrates that 39% of prostate cancers demonstrate nuclear staining over and above the cytoplasmic staining seen in normal prostate tissue. The enhanced expression may result in increased WT1 peptide presentation in prostate cancer, facilitating T cell recognition. However, WT1 expression in normal prostate tissue, as well as in other normal tissues, raises the issue of immunological tolerance to WT1. This might hinder the generation of effective WT1 T cell responses *in vivo*. Nonetheless, previous work in our laboratory and by others has shown that tolerance to WT1 is incomplete, as WT1 T cell responses can be found in patients with WT1 expressing leukaemias and solid tumours (160;161).

Using an HLA-A*0201/pWT126 tetramer I have characterised the T cell immune response to WT1 in prostate cancer patients. The HLA-A2 allele is expressed by approximately 40-50% of the Caucasian population, with approximately 95% of HLA-A2 individuals expressing the HLA-A*0201 subtype (162;163). In pilot experiments, I validated the pWT126/HLA-A*0201 tetramer to determine its specificity and sensitivity.

3.2.2.1 Validation of the pWT126/HLA-A*0201 tetramer

3.2.2.1.1 Background staining with the tetramer in healthy controls

A panel of peripheral blood mononuclear cells (PBMC) form healthy HLA-A2-positive donors was used to assess the level of background staining of the tetramer reagent. PBMC were separated from whole blood which was obtained from the National Blood Service and HLA-A2 status was confirmed by anti-A2 antibody staining and FACS analysis. The PBMC were stained with anti-CD3 and anti-CD8 antibodies, and PI was used to gate out dead cells. For each PBMC sample, the same number of cells were stained with anti-CD3 (FITC) and anti-CD8 (APC) antibodies in the absence and in the presence of PE-labelled tetramer. The flow cytometric analysis of the sample without tetramer was used to set the quadrants such that none of the viable CD3+CD8+ T cells recorded as PE-positive (Figure 3.2). These quadrants were then

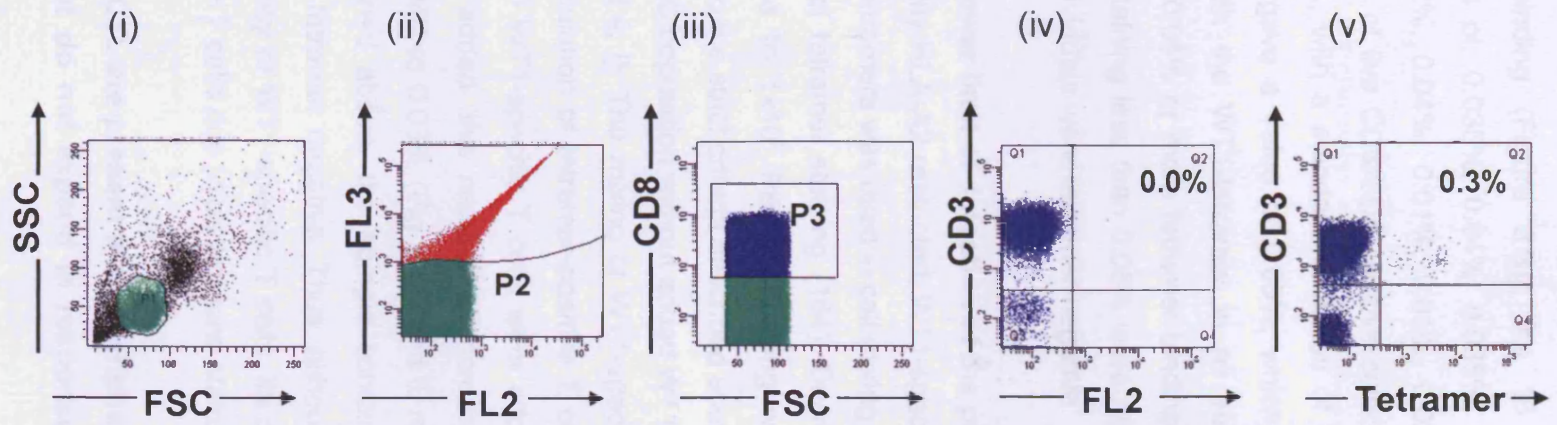

Figure 3.2 FACS gating strategy:

PBMC were stained with tetramer-PE, anti-CD3 and anti-CD8 antibodies. Propidium iodide (PI), which gives a signal in FL2 and FL3, was then added to differentiate dead from viable cells. Cells were then analysed on an LSR-II.

(i) On the FSC/SSC dot plot, a lymphocyte gate was assigned: P1

(ii) P2 is the live lymphocyte gate: cells which stain with PI are excluded

(iii) The P3 gate includes live, CD8 positive lymphocytes

(iv) Quadrants are set on CD3/CD8 positive cells (P3) in the absence of tetramer staining such that Q2 contains 0.0% CD3/CD8 positive cells

(v) Tetramer analysis is performed on P3. CD3 positive, tetramer positive cells are seen in Q2 and expressed as a percentage of total CD3/CD8 positive cells (Q1+Q2).

used for the analysis of the sample stained with tetramer to record the number of viable CD3+CD8+ T cells that bound tetramer. The analysis of sixteen normal PBMC samples revealed that no more than 0.04% of viable CD3+CD8+ T cells showed tetramer binding (Figure 3.3). The 16 normal blood samples had tetramer frequencies of: 0.035%, 0.04%, 0.035%, 0.014%, 0.015%, 0.03%, 0.0%, 0.04%, 0.0%, 0.02%, 0.04%, 0.01%, 0.01%, 0.02%, 0.03% and 0.0%, expressed as a percentage of live CD3/CD8 positive cells. The mean of the tetramer-positive cells was 0.02%, with a standard deviation of 0.015. Thus, the mean plus 2 standard deviations gave a value of 0.05%, which was used as a cut-off for background staining with the WT1-tetramer in all subsequent experiments. PBMC samples containing 0.05% or more tetramer binding cells were considered as positive, while PBMC containing less than 0.05% were negative. Using this cut-off, all PBMC from healthy individuals were tetramer-negative.

3.2.2.1.2 Lower limit of detection of the pWT126/HLA-A*0201 tetramer

A high avidity HLA-A2 restricted, WT1-specific T cell line that showed bright staining with WT1-tetramers was used in cell mixing experiments with PBMC to determine the sensitivity of tetramer staining (164). Decreasing numbers of WT1-specific T cells were added to $1x10^6$ PBMC. Staining was performed and quadrants were set according to the strict criteria described above. Background staining with the tetramer in the PBMC population without added WT1-specific cells was 0.01% of $CD3^+CD8^+$ T cells (Fig 3.4, i). The mixing of WT1-specific T cells with the PBMC resulted in a distinct population of tetramer-positive T cells that was readily detectable when as few as 150 WT1-specific T cells were added. Even when only 80 WT1-specific T cells were added, this resulted in increased tetramer staining from 0.01% in the control PBMC to 0.03% (Fig 3.4, i and ii, respectively). However, using the 0.05% cut-off defined above, the sample containing 80 WT1-specific T cells would be defined as tetramer negative. Thus, although the WT1-tetramer can identify a very low frequency of WT1-specific T cells, its detection limit was set at 0.05% of viable CD3+CD8+ T cells due to background staining in normal PBMC.

3.2.3 WT1 CTL are present in the peripheral blood of 53% patients with prostate cancer, but do not expand in response to peptide specific stimulation with IL2/IL7

Peripheral blood was obtained from 100 prostate cancer patients after obtaining written informed consent. Of the 100 patients, 38 were HLA-A2-positive as determined by flow cytometry. The clinical characteristics of these patients are listed

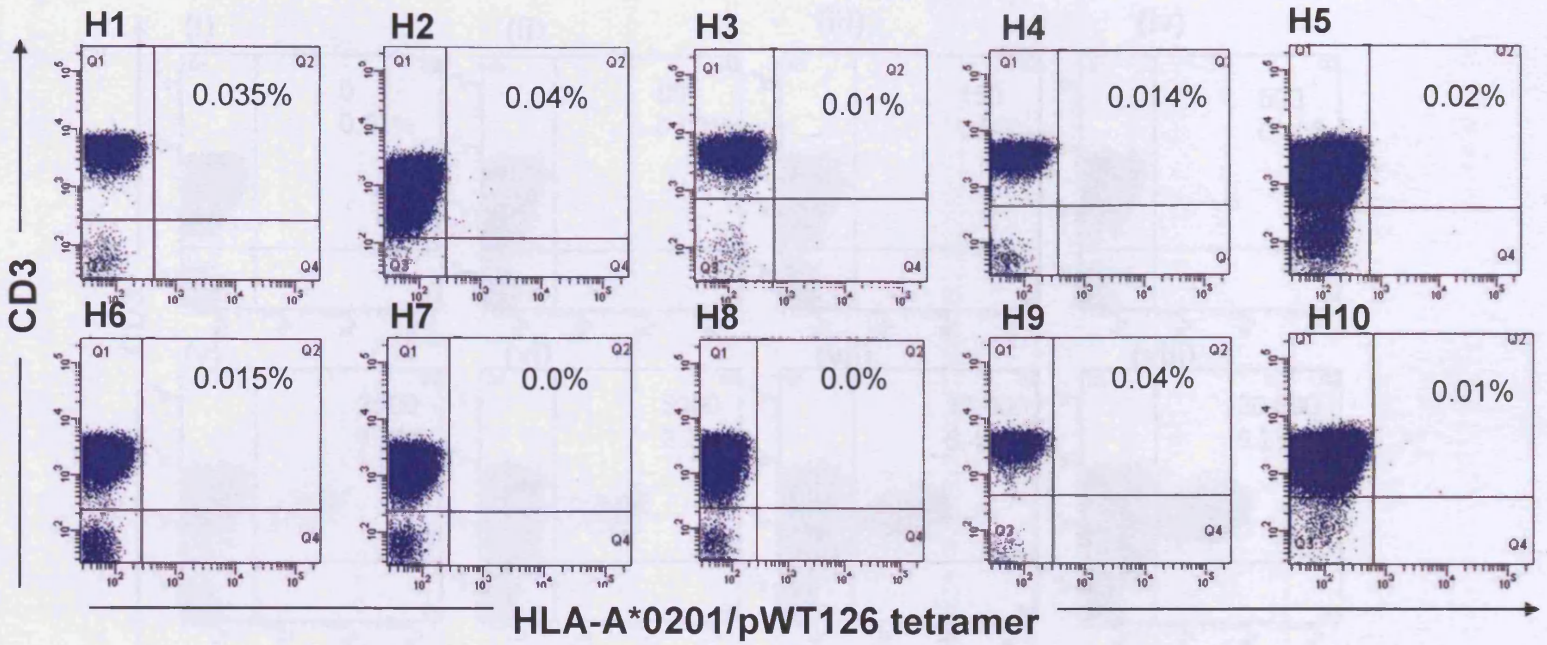

Figure 3.3 Background staining with the pWT126/HLA*0201 tetramer in HLA A2 positive healthy controls
PBMC were stained with HLA-A*0201/pWT126 tetramer, anti-CD3 and anti-CD8 antibodies and propidium iodide (PI). Dot plots display gated CD8+ live lymphocytes. The percentage of CD8+CD3+ positive T cells that bound the tetramer is indicated in each plot. All PBMC samples were first stained with anti-CD3, anti-CD8 and PI in the absence of tetramer to set the quadrants such that no events were recorded in the top right quadrant. Following tetramer staining, the top right quadrant was used to record tetramer binding T cells. HLA-A2 positive PBMC from healthy donors (H1-H10) were stained *ex vivo*. In total 16 healthy donors were analysed, and the mean percentage of tetramer binding cells was 0.02%, with a standard deviation of 0.015. The mean plus 2 standard deviations (=0.05%) was used as cut off for 'background' tetramer binding.

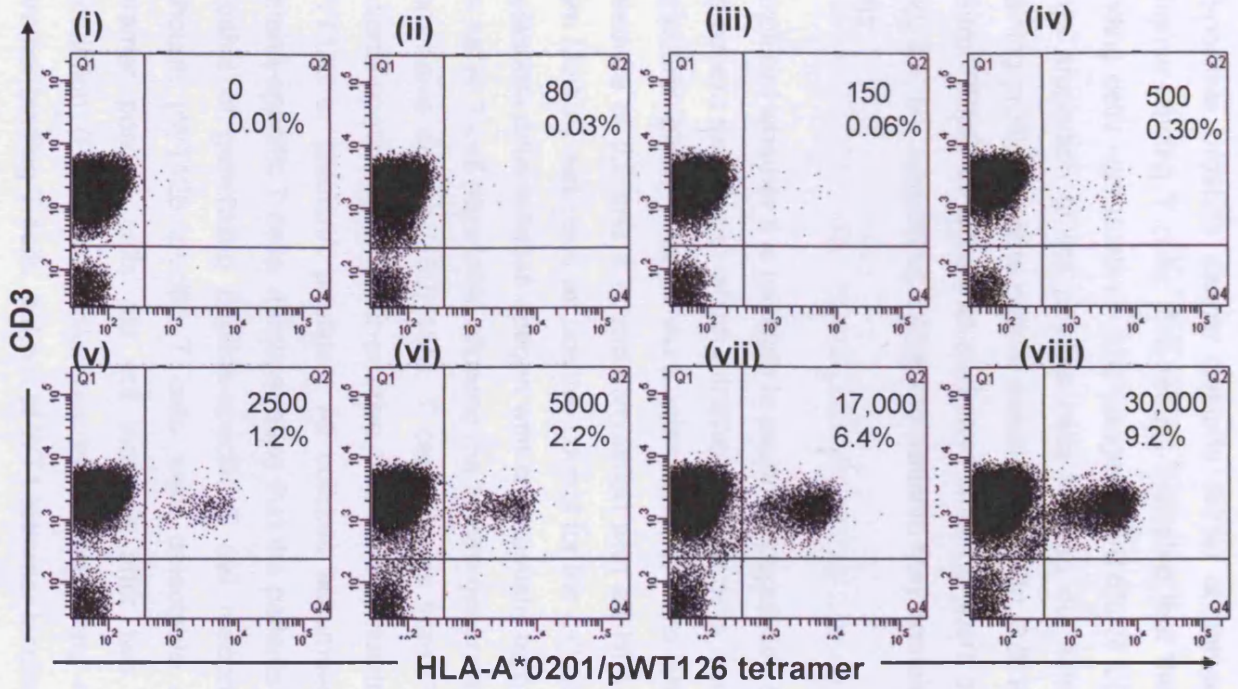

Figure 3.4: Validation of the pWT126/HLA-A*0201 tetramer

Mixing experiments using PBMC and a WT1-specific T cell line were used to determine the detection limit of WT1 tetramer staining. i) staining profile of PBMC without mixed WT1 specific T cells defined the tetramer background binding as 0.01%. ii-viii) show the tetramer staining profile of 10^6 PBMC mixed with 80-30,000 WT1-specific T cells. PBMC were stained with HLA-A*0201/pWT126 tetramer, anti-CD3 and anti-CD8 antibodies and propidium iodide (PI). Dot plots display gated CD8+ live lymphocytes. The percentage of CD8+CD3+ positive T cells that bound the tetramer is indicated in each plot. All PBMC samples were first stained with anti-CD3, anti-CD8 and PI in the absence of tetramer to set the quadrants such that no events were recorded in the top right quadrant. Following tetramer staining, the top right quadrant was used to record tetramer binding T cells.

in Table 3.2. Tetramer staining experiments were performed with all HLA-A2-positive patient samples applying the conditions that were used in the validation experiments described above. Figure 3.5 shows representative FACS plots of the tetramer staining profiles of 10 patients. Six patient samples scored positive as 0.05% or more of the CD3+CD8+ T cells bound the WT1-tetramer, while the remaining 4 patient samples did not show tetramer binding above background. In total, 20 out of 38 HLA-A2-positive prostate cancer patients (53%) contained detectable frequencies of tetramer-binding T cells. The results indicated that the frequency of WT1-tetramer binding cells was relatively low (range 0.05-0.6% of $CD3^+CD8^+$ T cells), and that a large proportion of the positive cells showed dull tetramer binding. However, the staining profile of some patient samples (e.g. Fig. 3.5, P5 and P6) was similar to the control PBMC samples that contained small numbers of added WT1-specific T cells (Fig. 3.4, iii), suggesting that some patients may contain high avidity WT1-specific T cells.

I explored whether it is possible to expand a population of tetramer positive cells from the patient samples in which tetramer-positive cells were detectable *ex vivo*. Patient peripheral blood T cells were stimulated *in vitro* with pWT126 peptides in the presence of IL2 and IL7, and stimulation with an HLA-A2-binding peptides derived from MelanA was used as positive control for the cell culture conditions. MelanA is a melanoma differentiation antigen with an unusually high T cell precursor frequency in the naïve T-cell repertoire, allowing the *in vitro* generation of MelanA -specific T cells from naive donors (160)·(165). T cell cultures from PBMC of 20 prostate cancer patients containing tetramer-positive cells were stimulated weekly for two rounds with pWT126 or pMelanA peptides. All cultures accumulated a distinct population of MelanA-specific T cells, demonstrating that the patients were immunocompetent and capable of generating peptide-specific T cell responses (Figure 3.6). However, although pWT126-specific T cells were detectable *ex vivo*, the percentage of tetramer positive cells did not increase after two rounds of peptide specific stimulation (Fig 3.7). Similar results demonstrating an accumulation of MelanA tetramer binding T cells, but not of WT1 tetramer binding cells were obtained with all 20 HLA-A2-positive patient samples, suggesting a selective inability of the WT1-specific cells to respond to peptide stimulation. This T cell unresponsiveness correlated with the results of ELISPOT assays, showing that pWT126 stimulation did not result in peptide-specific IFNγ production (Figure 3.8). Together, these data suggested that the WT1-specific T cells in prostate cancer patients were tolerant and unable to proliferate or produce IFNγ in response to peptide specific stimulation.

Table 3.2: clinical characteristics of HLA-A2 prostate cancer patients

Patient	Age	Stage*	Prior therapy	Gleason grade**	WT1 tetramer (% CD3+CD8+)
1	84	T3NxM1	Androgen ablation	-	0.6
2	69	T1N0M0	Radiotherapy, androgen ablation	8	0.06
3	78	T2N0M0	Radiotherapy, androgen ablation	6	0.02
4	76	T3NxM1	Radiotherapy, androgen ablation	7	0.05
5	60	T3N0M0	Surgery, radiotherapy	6	0.08
6	79	T3N0M0	Radiotherapy, androgen ablation	-	0.5
7	73	T2N0M0	Radiotherapy	7	0.02
8	69	T1N0M0	Radiotherapy	7	0.02
9	64	T3NxM1	Androgen ablation	-	0.15
10	68	T2N0M0	Radiotherapy, androgen ablation	6	0.03
11	64	T3NxM1	Androgen ablation	9	0.13
12	73	T2N0M0	Radiotherapy, androgen ablation	6	0.1
13	56	T2N0M0	Radiotherapy, androgen ablation	6	0.03
14	67	T2N0M0	Radiotherapy	6	0.02
15	78	T1N0M0	Androgen ablation	7	0.04
16	82	T2N0M0	Androgen ablation	7	0.04

17	81	T3NxM0	Radiotherapy	-	0.04
18	67	T1N0M0	Radiotherapy	7	0.03
19	83	T2N0M0	Surgery	-	0.08
20	64	T2N0M0	Radiotherapy, surgery	6	0.1
21	82	T2N0M0	Surgery	7	0.6
22	68	T1N0M0	Surgery	6	0.2
23	64	T3N0M0	Radiotherapy, androgen ablation	7	0.08
24	71	T2N0M0	Surgery	6	0.03
25	81	T3N1M1	Androgen ablation	8	0.01
26	66	T2N0M0	Radiotherapy	-	0.07
27	82	T2N0M0	Radiotherapy, androgen ablation	7	0.07
28	80	T2N0M0	Radiotherapy, androgen ablation	6	0.03
29	74	T2N0M0	Radiotherapy, androgen ablation	7	0.01
30	69	T3N0M0	Radiotherapy	-	0.07
31	64	T3N1M0	Radiotherapy, androgen ablation	9	0.02
32	82	T3NxM1	Androgen ablation	7	0.08
33	55	T2N0M0	Radiotherapy, androgen ablation	6	0.03
34	73	T2N0M0	Radiotherapy	6	0.04

35	50	T2N0M0	Radiotherapy, surgery	8	0.1
36	84	T3N1M1	Radiotherapy, androgen ablation	7	0.07
37	96	T3N0M0	Androgen ablation	7	0.06
38	82	T2N0M0	Androgen ablation	7	0.02

* TNM: tumour, nodes, metastases staging (Nx: information not available)

** Gleason: degree of differentiation seen histologically (2 scores from 0-5, with 5 representing poorly differentiated tumours)

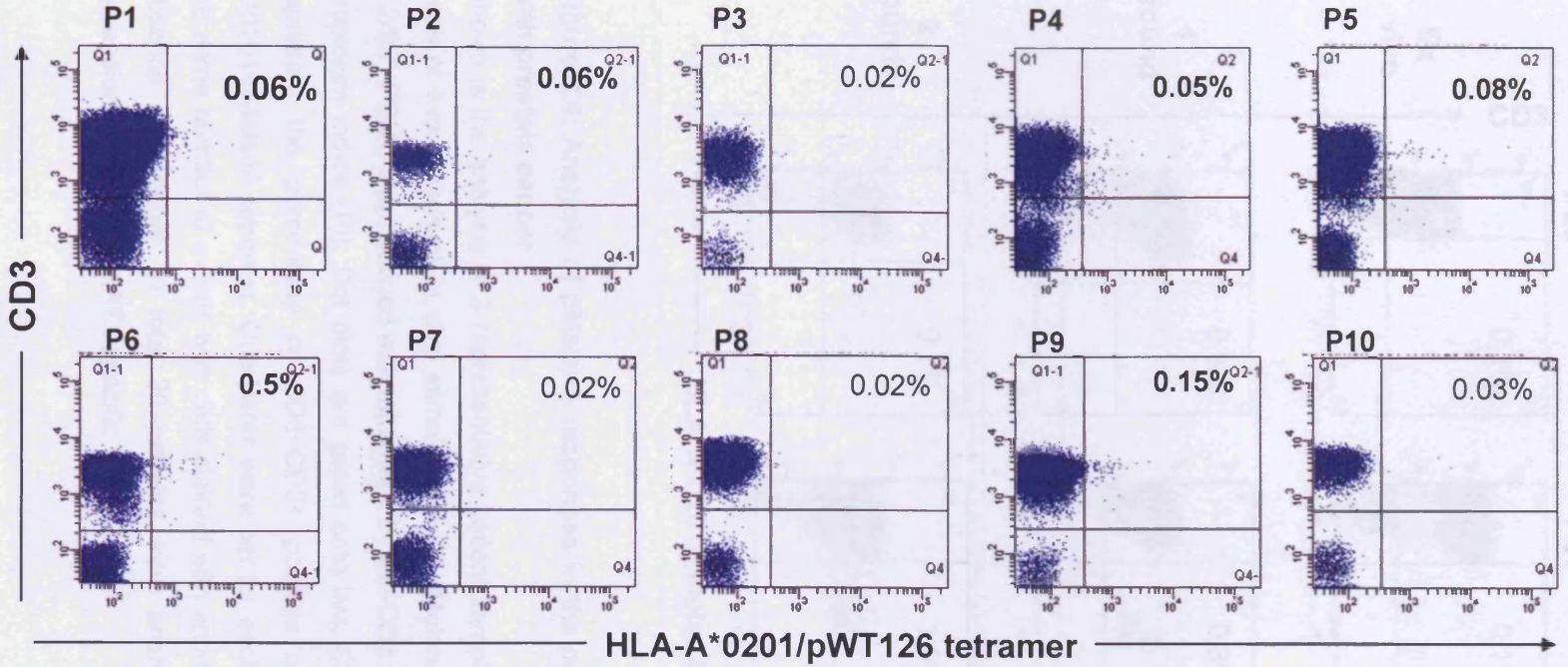

Figure 3.5: WT1 tetramer staining of PBMC of prostate cancer patients

Shown are representative tetramer staining profiles of 10 patients; the percentages in bold indicate positive tetramer staining above background. In total 38 HLA-A2 patients were analysed, and 20 patients (53%) demonstrated tetramer binding (range: 0.05-0.6%). PBMC were stained with HLA-A*0201/pWT126 tetramer, anti-CD3 and anti-CD8 antibodies and propidium iodide (PI). Dot plots display gated CD8+ live lymphocytes. The percentage of CD8+CD3+ positive T cells that bound the tetramer is indicated in each plot. All PBMC samples were first stained with anti-CD3, anti-CD8 and PI in the absence of tetramer to set the quadrants such that no events were recorded in the top right quadrant. Following tetramer staining, the top right quadrant was used to record tetramer binding T cells

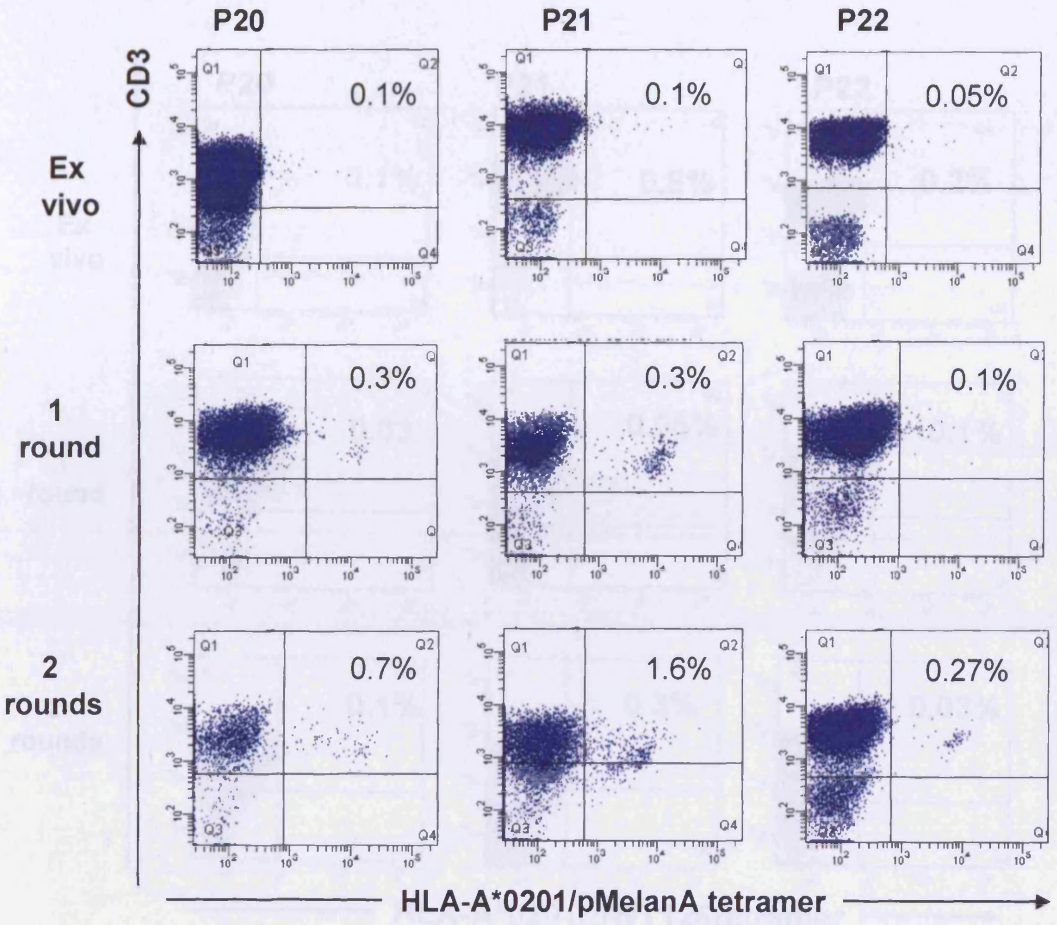

Figure 3.6: Analysis of pMelanA responses in the peripheral blood of patients with prostate cancer

Shown is the analysis of 3 representative patient samples stained *ex vivo* and after each of two rounds of *in vitro* stimulation with pMelanA/MART1in the presence of IL2/IL7. PBMC were stained with tetramer-PE, anti-CD3 and anti-CD8 antibodies and propidium iodide (PI). Dot plots are gated onto live, CD8+ lymphocytes. Numbers represent the percentage of CD8+CD3+ positive cells that bound the HLA-A*0201/pMelanA tetramer. Quadrants were set for each experiment after collecting the same number of events with cells stained with anti-CD3, anti-CD8 and PI in the absence of tetramer. In total 20 patients were analysed, all of whom showed expansion of MelanA/MART1 specific T cells.

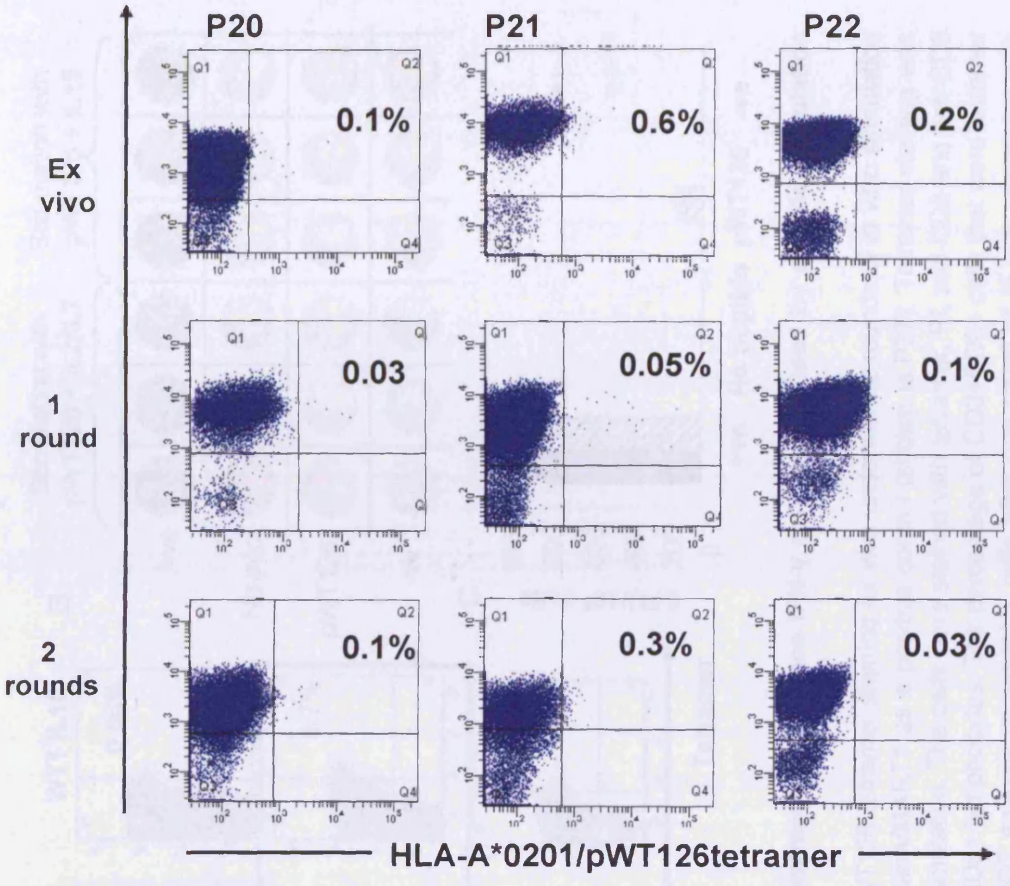

Figure 3.7: Analysis of pWT126 responses in the peripheral blood of patients with prostate cancer

Tetramer staining was performed ex vivo and after each of two rounds of in vitro stimulation with pWT126 in the presence of IL2/IL7. PBMC were stained with tetramer-PE, anti-CD3 and anti-CD8 antibodies and propidium iodide (PI). Dot plots are gated onto live, CD8+ lymphocytes. Numbers represent the percentage of CD8+CD3+ positive cells that bound the HLA-A*0201/pMelanA tetramer. Quadrants were set for each experiment after collecting the same number of events with cells stained with anti-CD3, anti-CD8 and PI in the absence of tetramer. 3 representative patients are shown (n=20).

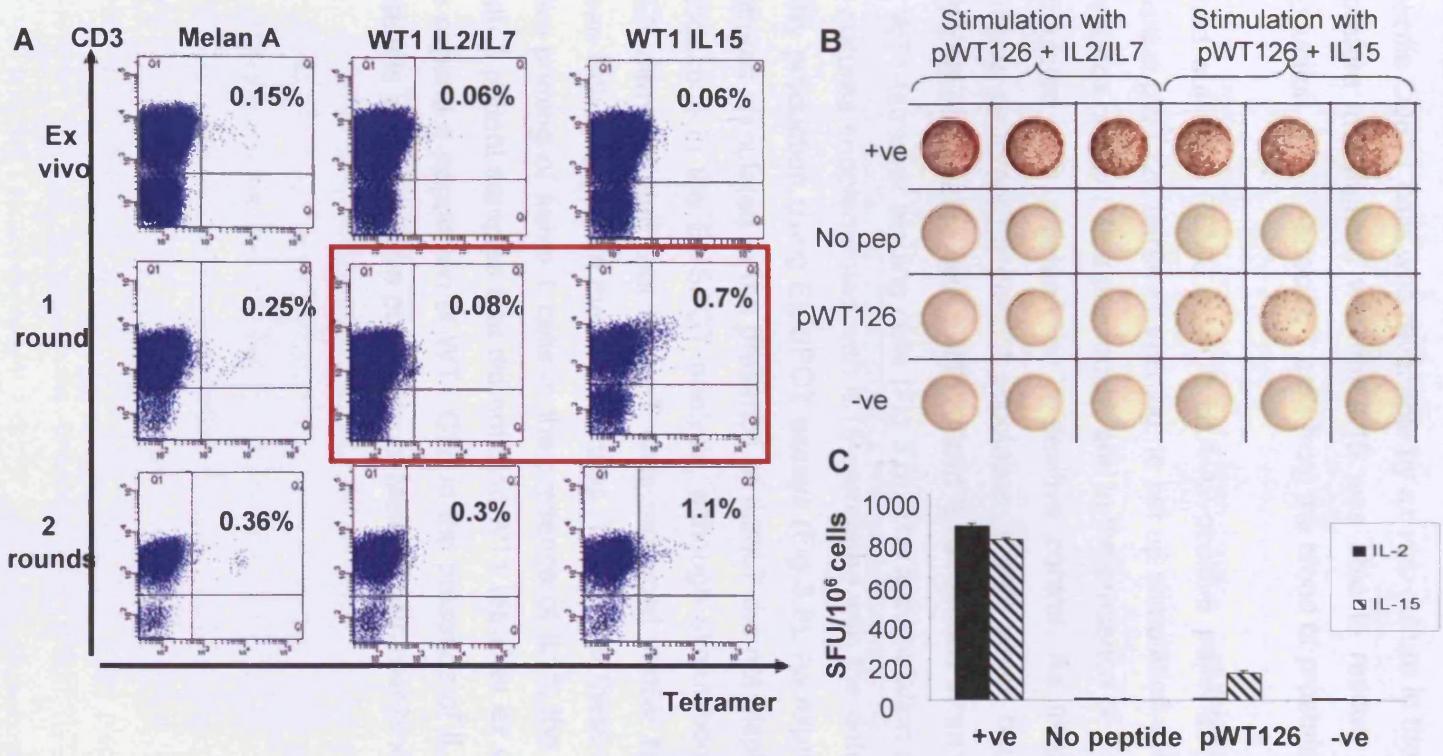

Fig. 3.8. WT1-specificT cells that bind tetramer and produce IFN-γ can be expanded by peptide stimulation with IL15, but not IL2/IL7.

(A) Patient samples which demonstrated pWT126 tetramer staining *ex vivo* underwent 2 rounds of *in vitro* stimulation with pWT126 and IL2/IL7 or IL15, and pMelanA/MART1 as a positive control (shown is P37). Tetramer staining was performed *ex vivo* and after each round of stimulation. The cells were stained with tetramer, PI, anti-CD8 and anti-CD3 antibodies; the dot plots show gated viable CD8+ lymphocytes. The percentage of CD3+CD8+ cells that were tetramer positive is indicated in each plot. (B) ELISPOT analysis of patient T cells after one round of *in vitro* expansion with pWT126 in the presence of IL2/IL7 or IL15 (phenotype indicated by red rectangle). T cells were stimulated overnight with autologous PBMC in the presence or absence of pWT126 peptides. As a positive control, T cell stimulation was performed with autologous PBMC and SEB. Negative control: responder T cells only. (C) The ELISPOT results are illustrated in a column graph (SFU: spot forming units).

3.2.4 The unresponsive phenotype of WT1-specific T cells can be reversed by *in vitro* culture with IL15

It has previously been shown in a murine model that tolerance of tumour antigen-specific $CD8^+$ T cells was reversible by *ex vivo* culture in the presence of IL15 (159). I therefore investigated whether IL15 was able to rescue the expansion and IFNγ production of WT1-specific T cells from the blood of prostate cancer patients.

I had sufficient PBMC from 8 HLA-A2-positive patients, which had demonstrated positive pWT126 tetramer staining, to set up stimulation cultures with pWT126 in the presence of IL2/IL7 as previously, and in the presence of IL15. The PBMC were also stimulated with pMelanA as a positive control. As in previous experiments, all patients generated a distinct population of cells which bound pMelanA tetramer. In 3/8 patients, culture with pWT126 and IL15 resulted in an increase in the frequency of WT1-tetramer binding cells (Fig 3.8). The accumulation of tetramer binding T cells in cultures supplemented with IL15 correlated with the detection of pWT126-specific IFNγ production using ELISPOT assays (Fig 3.8). As expected, the T cells from the cultures stimulated in the presence of IL2/IL7 did not display pWT126-specific IFNγ production in the ELISPOT analysis, although stimulation with the positive control SEB demonstrated that these T cells produced similar numbers of IFNγ spots as seen with T cells from the IL15 cultures. To exclude these results being a result of *in vitro* priming of naïve T cells in the presence of IL15, the experiment was repeated with 5 patient samples that did not bind WT1 tetramer *ex vivo*. All five patients failed to expand a population of WT1 CTL in the presence of IL2/IL7 or IL15, although all patients accumulated a population of Melan A tetramer binding T cells (Fig 3.9).

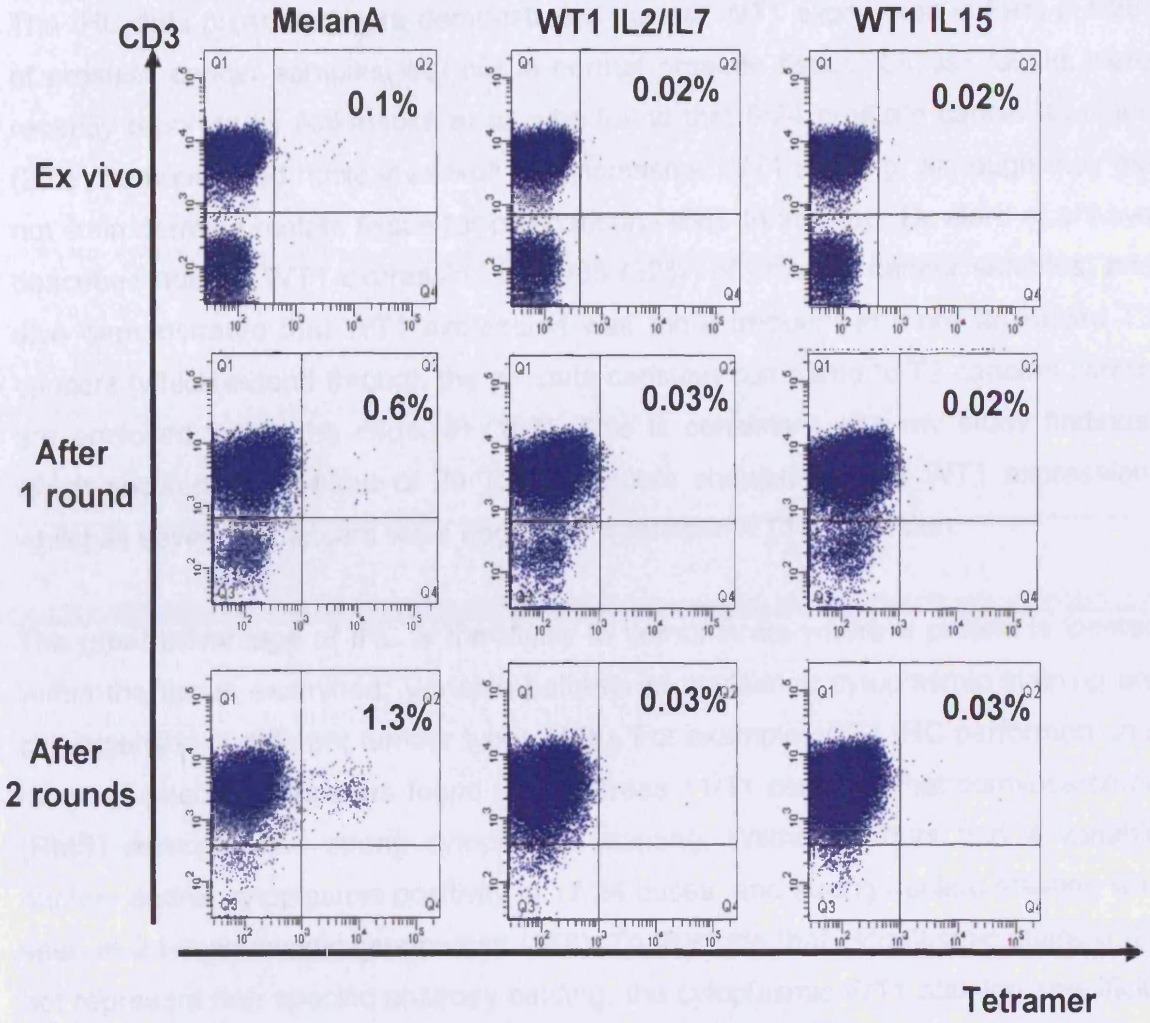

Figure 3.9: patients in which pWT126-specific T cells were not detected *ex vivo* did not expand a population of tetramer binding cells following in vitro culture with either IL-2/IL-7 or IL-15. Patient samples which did not demonstrate pWT126 tetramer staining ex vivo underwent 2 rounds of in vitro stimulation with pWT126 and IL2/ IL7 or IL15. Antigen stimulation with pMelan A was used as a positive control for cell culture conditions. Tetramer staining was performed ex vivo and after each round of stimulation: cells were stained with PE-labelled tetramer, propidium iodide, anti-CD8 and anti-CD3 antibodies and analysed by flow cytometry. The FACS plots display gated viable CD8+ lymphocytes. Figures represent the percentage of viable CD3+CD8+ cells that are tetramer positive. Representative results from one patient (P32) are shown (n=5 patients). pMelan-A-specific T cells were detectable, after 2 rounds of peptide-specific stimulation, in all samples.

3.3 Discussion

The IHC data presented here demonstrated nuclear WT1 expression in 39% (14/36) of prostate cancer samples, but not in normal prostate tissue. Similar results were recently reported by Nakatsuka et al, who found that 6/24 prostate cancer samples (25%) demonstrated nuclear as well as cytoplasmic WT1 staining, although they did not stain normal prostate tissue for comparison (166). In addition, Devilard *et al* have described nuclear WT1 expression in 44/85 (52%) of prostate cancer samples, and also demonstrated that WT1 expression was more frequent in more advanced T3 cancers (which extend through the prostate capsule) compared to T2 cancers (which are enclosed within the capsule) (167). This is consistent with my study findings, which showed that 14 out of 29 T3/T4 cancers showed nuclear WT1 expression, whilst all seven T2 cancers were negative for nuclear WT1 expression.

The great advantage of IHC is the ability to demonstrate where a protein is located within the tissue examined. Variable patterns of nuclear or cytoplasmic staining are characteristic of different tumour types (166). For example, WT1 IHC performed on a range of paediatric tumours found that whereas 11/11 cases of rhabdomyosarcoma (RMS) demonstrated strong cytoplasmic staining, Wilms' tumours had a variable nuclear and/or cytoplasmic positivity in 17/24 cases, and strong nuclear staining was seen in 2 lymphoblastic lymphomas (168). To illustrate that cytoplasmic staining did not represent non specific antibody binding, the cytoplasmic WT1 staining specificity in RMS was also confirmed by Western blot analysis and RT-PCR.

WT1 expression in normal prostate epithelial cells was shown to be confined to the cytoplasm, suggesting that the regulation of WT1 is altered in prostate cancer. A recent study has also described cytoplasmic WT1 expression in normal lung epithelial cells (169). In this case, the exposure of lung epithelial cells to inflammatory cytokines such as tumour necrosis factor alpha (TNFα) resulted in the translocation of WT1 from the nucleus to the cytosol. Phosphorylation of residues in the zinc finger domain of WT1 protein by protein kinase A was shown to disrupt DNA binding and result in the cytoplasmic accumulation of WT1 (170;171). Although WT1 was originally described as a nuclear transcription factor, it has more recently been shown to shuttle between the nucleus and the cytoplasm, and it has been suggested that WT1 also plays a role in post-transcriptional regulation (172). Structural modeling has revealed a potential RNA recognition site in the N-terminus of WT1 (173), and it was also demonstrated that the zinc-finger domain in the C-terminal part of WT1 could

bind to RNA (174). In addition, cytoplasmic WT1 is present within ribonucleoprotein particle (RNP) complexes, and is associated with ribosomes and actively translating polysomes. This data lends weight to the idea that WT1 also plays a role in post-transcriptional processes (172).

The WT1 gene products exist in over 25 isoforms. These have a range of functions and may act as an activator or repressor of genes under different circumstances. There are a number of mechanisms by which the function of the WT1 protein may be modulated, including phosphorylation, dimerization and alternative splicing. In mammals, exons 5 and 9 of WT1 are alternatively spliced, giving rise to four different splice isoforms (103;175). The +KTS/−KTS ratio has been shown to be strongly conserved (176). The importance of the ratios is demonstrated in Frasier syndrome, which results from a mutation in the splice donor site in intron 9 of one *WT1* allele, resulting in the loss of expression of the WT1+KTS form of one allele (177). The change in ratio of +KTS/−KTS is responsible for the severe developmental defects in this disease. Silberstein *et al* have also demonstrated that the proportion of different splice variants was different in breast cancer samples compared to normal breast tissue, suggesting a mechanism for WT1's involvement in breast cancer development through altered WT1 isoform ratios (178)

The role of WT1 in the oncogenesis of prostate cancer has yet to be established. However, it is known that WT1 interacts with prostate apoptosis response gene, Par4 (179). Furthermore, Cheema *et al* demonstrated in prostate cancer cell lines that Par4 regulates proto-oncogene bcl-2 expression through a WT1 binding site on the bcl-2 promoter; bcl-2 being known to be associated with the development of androgen resistance in prostate cancer (180). They observed decreased bcl-2 expression in prostate cancer cell lines in response to treatment with all trans-retinoic acid (ATRA) associated with a rise in WT1 expression, and also showed that bcl-2 expression could be increased by blocking WT1. However, WT1 is known to bind to two different regions on the bcl-2 promoter, and can regulate bcl-2 transcription by acting as either an activator or a repressor (181) (182), so it is not yet clear how these *in vitro* results relate to the physiological situation. The suggestion that the loss of WT1 correlates with the development of androgen resistance also contradicts data presented by Devilard *et al*, who report that WT1 gene expression levels were associated with androgen independent prostate cancer in human prostate cancer samples (167).

While the advantage of immunohistochemistry is the ability to demonstrate the location of the protein under investigation, this technique also has a number of limitations. Firstly, immunohistochemistry is not quantitative. Although researchers can assign a figure from 0 (no staining) to 3 (very strong staining), this itself is subjective and researcher dependent. Tissue handling and fixation techniques can also affect the quality of the samples and the sensitivity of subsequent IHC assays. Western Blot is another method of measuring protein levels which is semi-quantitative. An alternative, quantitative, assay to measure WT1 expression is real-time PCR, whereby the amount of mRNA is determined by comparing the results to a standard curve produced by performing PCR on known amounts of DNA.

A further issue with immunohistochemistry is that of antibody cross reactivity. The staining protocol therefore includes several important controls. When the antibody was validated, skeletal muscle was used as a negative control. As a further negative control, the primary antibody is replaced with assay diluent, to exclude non-specific binding by the secondary antibody or other reagents. It was previously thought that cytoplasmic immunohistochemistry staining might represent non specific antibody binding, but it has since been shown that WT1 cytoplasmic staining correlates with Western Blot and RT-PCR data (167;168). In addition, in my series of prostate cancer samples there are samples which do not stain in the cytoplasm or the nucleus, which act as an internal negative control, since one would expect any cross reactivity to be present in all prostate samples. Whether or not the cytoplasmic WT1 staining of normal prostate tissue seen in Figure 3.1 represents true staining or non specific staining may have implications for toxicity to normal prostate tissue that might result from immunotherapeutic strategies which target WT1. This could be further investigated by Western Blot or RT-PCR analysis of normal prostate tissue, to look for WT1 protein or mRNA, respectively.

The majority of peptides presented by MHC Class I molecules are derived from cytosolic or nuclear proteins. In those cases where prostate cancer cells express WT1 in the nucleus in addition to that seen in the cytoplasm, this may result in increased presentation of WT1 peptides on the surface of these prostate cancer cells, which could facilitate T cell recognition. However, to address this, mass-spectrometry and sequence analysis of peptides eluted from fresh tumour material would be required (183).

I found a low frequency of WT1-tetramer binding T cells in 53% of prostate cancer patients, suggesting that tumour cells had stimulated autologous T cell responses. It would have been interesting to correlate IHC staining with WT1 tetramer staining for individual patients. However, we did not have ethical approval for this. Furthermore, the original tissue blocks were often not available, since the majority of patients had been diagnosed elsewhere before being referred for radiotherapy.

My data is consistent with previously published literature reporting a low frequency of WT1-specific T cells in patients with WT1-expressing malignancies. Oka et al reported ex vivo tetramer staining in 2 breast cancer and 8 lung cancer patients, with a range of 0.08-1.31% of CD8s (116;160). Studies in leukaemia patients have demonstrated the presence of IFNγ-producing WT1-specific T cells in the peripheral blood (161;184). Why some patients possess WT1 CTL which bind tetramer, while others do not, is not well understood. There is increasing data that tetramer staining can occur in the absence of effector function, and vice versa (150;157;185;186). WT1 CTL may be present in the peripheral blood of prostate cancer patients at too low a frequency to be detected by tetramer staining. Rezvani *et al* used quantitative PCR to measure IFN-γ mRNA production in response to peptide stimulation as a surrogate for the presence of antigen specific CTL. They found this method to be approximately 10 times more sensitive than tetramer staining, and were able to detect pWT126-specific responses *ex vivo* in the peripheral blood of patients with chronic myelogenous leukaemia as well as that of healthy HLA-A2-positive blood donors (184).

Clinical studies have demonstrated that WT1-specific T cells are detectable in patients, and that they are associated with anti-tumour effects after vaccination and after allogeneic transplantation. An increase in the frequency of WT1-specific T cells following allogeneic stem cell transplantation correlated with a decrease in WT1 transcript frequency in the peripheral blood of leukaemia patients, suggesting that WT1-specific CTL contributed to a reduction in the number of WT1 expressing leukaemia cells (187). Further evidence for the anti-tumour effect of WT1-specific CTL has been obtained in vaccination studies in patients with leukaemia and solid tumours. A correlation was found between vaccine induced T cell responses and a reduction in tumour burden, tumour markers or WT1 transcript numbers (116;188). Conversely, the loss of WT1-specific T cells was shown to correlate with the reappearance of WT1 expressing leukaemia cells (196).

However, in my study, the naturally occurring WT1 specific T cells in prostate cancer patients were functionally impaired and unable to expand upon WT1-peptide stimulation in the presence of IL2 and IL7. A similar observation was made recently in patients with leukaemia, when naturally occurring WT1-specific T cells failed to expand upon WT1-peptide stimulation in the presence of IL2 and IL7. In this study, the removal of the CD4+CD25+ T cell population restored the ability of WT1-specific T cells to expand (189). In my study, IL15 was able to restore the functional activity of WT1-specific T cells in 3 out of 8 patients tested. It is possible that regulatory CD4+CD25+ T cells were responsible for T cell suppression in those patients that did not respond to IL15.

The induction of T cell unresponsiveness has been described in melanoma patients and in murine tumour models (12;156;157). Although immunogenic tumours stimulated antigen-specific T cell responses, these T cells were rendered non-functional and unable to control tumour growth (12). In another study the adoptive transfer of T cells initially resulted in tumour regression, but this was followed by progressive tumour growth associated with the induction of unresponsiveness in the transferred T cells (190). There are extensive reports of T cells isolated from patients with tumours being functionally impaired (191). In such cases, tetramer staining may not correlate with the presence of functional cells and may be misleading in cancer patients, where tumour antigen specific T cells have been subject to peripheral tolerance mechanisms.

Considering the similarity of the cytokine receptors for IL2 and IL15, it is surprising that IL15 restored the functional activity of tolerant T cells that did not respond to IL2. The IL15 receptor is one of at least six different interleukin receptors (IL2, IL-4, IL7, IL-9, IL15 and IL21) to which the gamma chain (γ_c) (CD132) cytokine receptor sub-unit is common. In addition, the receptor complexes for IL2 and IL15 both share a β (CD122) subunit, forming a medium affinity $\beta\gamma_c$ receptor which results in functional overlap of IL2 and IL15, such as stimulating T cell proliferation (192;193). However, each cytokine also binds to a unique α chain which forms a high affinity receptor complex with the $\beta\gamma_c$ complex, giving rise to the different roles each cytokine plays in T cell biology (194). Signalling via the IL2 receptor α (Rα) (CD25) can result in activation induced cell death (195) and inhibit memory cell proliferation and survival (196), whereas signalling through the IL15 Rα complex is required for the development of components of the innate immune system, and for the maintenance of memory CD8 T cells (197;198). The IL15 Rα is the likely candidate for mediating

the effects seen with IL15 in this study, although the intracellular signalling pathways that lead to distinct biological responses upon IL2 and IL15 binding are currently not fully understood. In classical T cell anergy, IL2 was very effective in reversing T cell unresponsiveness (158). However, recent experiments in transgenic mice revealed that tolerance of T cells specific for a tumour-associated model antigen was reversed by IL15, but not IL2 (159). This study in prostate cancer patients is the first description of a similar situation in humans, where T cells specific for a tumour-associated antigen are unresponsive to IL2, but recover functional activity when stimulated in the presence of IL15. IL2 is routinely used to enhance the functional activity of adoptively transferred tumour-reactive T cells in vivo at present. The data described here raises the possibility that IL15 may promote T cell function in those patients who do not respond to treatment with IL2.

Chapter 4: The effect of additional CD3 on endogenous TCR expression in $58^{\alpha-\beta-}$ cells

4.1 Introduction

Retroviral TCR gene transfer is an attractive strategy by which large numbers of antigen specific T cells can be generated for adoptive transfer (133;135;199-202). One of the advantages of this technique is that it can be used to circumvent possible impairment of autologous T cell responses against tumour associated antigens, since central tolerance is bypassed. In addition, the introduced TCR specificity can be targeted against poorly immunogenic targets and a high avidity TCR can be selected for transfer. This method has recently seen success in the first clinical trial of TCR gene therapy, where MART1 specific T cells - generated by retroviral gene transfer - were adoptively transferred into patients with metastatic melanoma. The engineered T cells engrafted in 15 out of 17 patients, 2 of whom demonstrated long term tumour regression (137).

Since the density of TCRs on the surface of cells affects their functional avidity, there are a number of factors which may affect the success of this technique (203;204). Several different strategies have been developed to increase expression of the introduced TCR by manipulating its structure, for example by murinising the constant domains of the α and β chains, by introducing an additional disulphide bond into the constant regions of the introduced TCR (150;205;206). More recently, α and β chains have each been linked to the CD3ζ chain, which results in increased preferential pairing of TCR chains in the Jurkat T cell line.

TCR alpha and beta chains must assemble with the CD3 complex in order for the TCR to be expressed on the cell surface. The CD3 complex is comprised of 4 invariant chains: gamma, delta, epsilon and zeta. One epsilon and one delta chain form a largely extracellular heterodimer, as do one gamma and one epsilon chain, whereas two zeta chains form a homodimer which is largely intracellular. The TCR and CD3 chains assemble separately in the ER before coming together to form the TCR/CD3 complex (Figure 4.1). The zeta chains are the last component to be integrated before the complex is transport to the cell surface (122-124).

At present it is unclear whether TCR and CD3 components are present in equimolar concentrations in T cells, or whether one is in excess. It may be that TCRs,

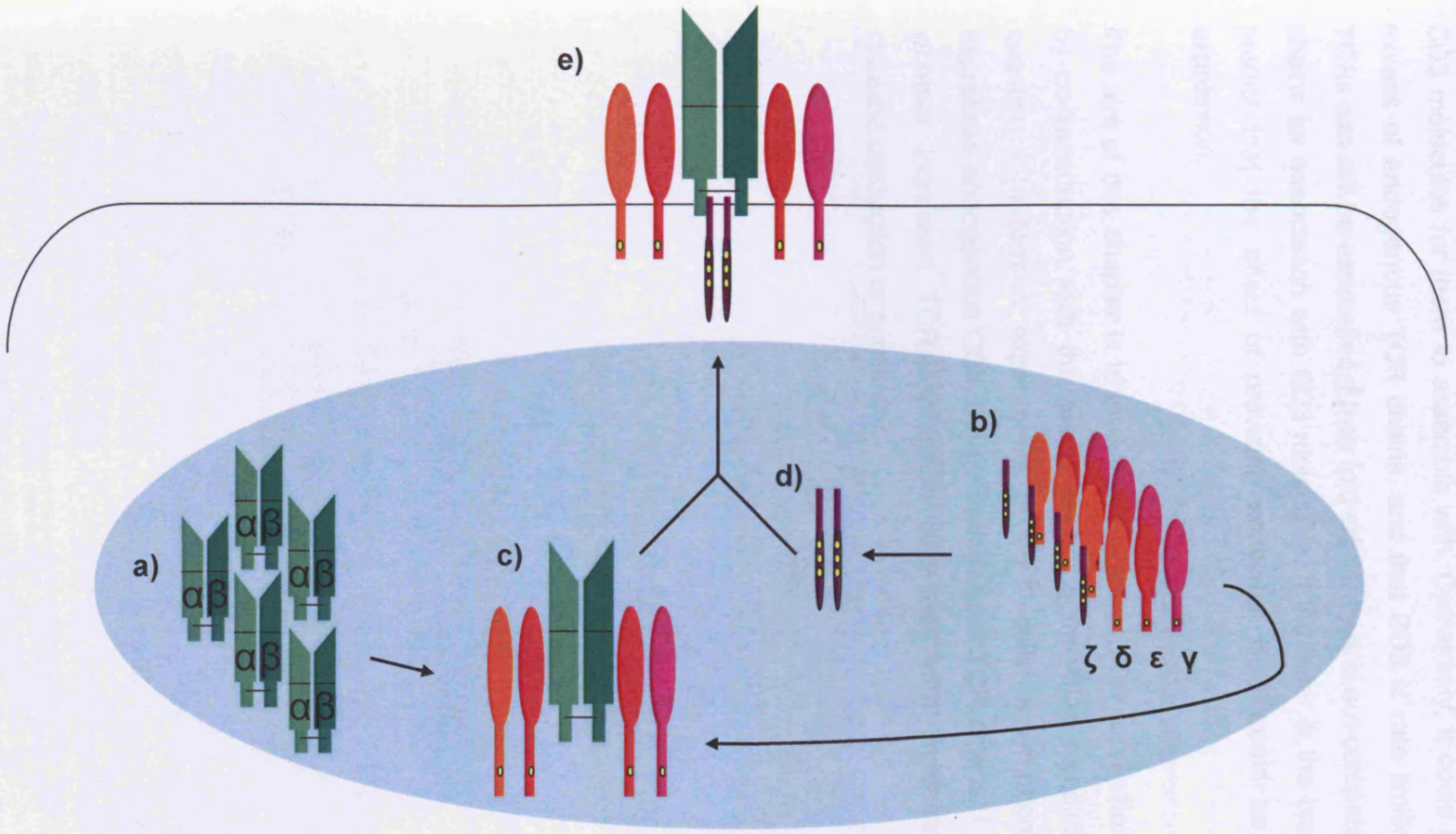

Figure 4.1: Assembly of the TCR/CD3 complex. a+b): The TCR α and β chains are transcribed and form a heterodimer in the endoplasmic reticulum, separately from the CD3ζ, δ, ε and γ chains. c) The TCR:CD3 complex subsequently assembles within the ER, with the CD3ζ chain being the last component to be integrated (d) before the complex is transported to the cell surface (e). Only complete TCR/CD3 complexes are displayed on the cell surface. Partially assembled TCRs are retained in the ER or targeted for degradation. It is not clear whether TCR and CD3 chains are produced in equimolar concentrations, or whether one of the two is in excess.

introduced by retroviral gene transfer, are expressed because there is an excess of CD3 molecules for them to assemble with. Conversely, it could be that there is an excess of endogenous TCR chains, and that CD3 is rate limiting, but exogenous TCRs can still be expressed if they fold efficiently and out-compete endogenous TCR chains for association with CD3 molecules. If the latter is the case, then one would predict that the effect of providing additional CD3 would be to increase TCR expression.

The aim of this chapter is to investigate whether TCR expression can be increased by co-transduction with the genes encoding the CD3 complex. To address this question I performed experiments in $58^{\alpha-\beta-}$ cells: a lymphoma cell line which expresses endogenous CD3, but no endogenous TCR chains. I have also explored whether increased TCR expression correlates with increased antigen specific cytokine production or sensitivity.

4.2 Results

4.2.1 CD3 is rate limiting for TCR expression in $58^{\alpha-\beta-}$cells

Initial experiments were performed in the $58^{\alpha-\beta-}$ cell line, a murine lymphoma cell line that expresses CD3, but no TCR α or β chains. Consequently, neither CD3 molecules nor TCR molecules are detectable on the surface of $58^{\alpha-\beta-}$ cells. The introduction of TCR α and β genes results in the assembly of TCR/CD3 complexes, which are expressed on the cell surface. I have explored whether the introduction of additional CD3 molecules can enhance TCR expression in this model system.

A schematic representation of the retroviral vectors used in these experiments can be found in Figure 4.2. The F5 TCR recognises a peptide from the nuclear protein of influenza, pNP366, in the context of H2-D^b Class I molecules. The WT1 TCR recognises a peptide from the human WT1 protein (pWT126) in the context of HLA-A*0201 Class I molecules. The WT1 TCR used in these experiments is a hybrid comprised of murine constant domains and human variable domains and is expressed in both murine and human cells. I have used this TCR because it is being investigated in the human system in the laboratory at present, and recent work has demonstrated that this hybrid WT1 TCR shows increased expression and tetramer binding in human T cells compared to the wild type, fully human WT1 TCR (150).

$58^{\alpha-\beta-}$ cells were transduced with a TCR alone or with additional CD3, using a retroviral construct containing the 4 CD3 chains in addition to GFP, hereafter referred to as CD3δ-GPF (Figure 4.2). GFP was then used to differentiate cells expressing only endogenous CD3 (GFP negative) from cells expressing additional exogenous CD3 (GFP positive). This set of experiments was repeated using the F5 TCR and the WT1 TCR. F5 TCR expression was assessed using the anti-Vβ-11 antibody and tetramer, WT1 TCR expression was assessed using the anti-Vβ-2.1 antibody and tetramer. $58^{\alpha-\beta-}$ cells were mock transduced as a control. As an additional control, $58^{\alpha-\beta-}$ cells were transduced with a retroviral GFP construct (hereafter referred to as GFP control) which contains the WT1 gene in reverse orientation, and from which only the GFP is expressed (Fig 4.2, d). The GFP control is required for flow cytometry to exclude the effects of GFP bleeding into the PE channel.

Mock transduced $58^{\alpha-\beta-}$ cells did not express surface CD3, Vβ-11 or bind tetramer (Figure 4.3, a-d). After transduction with F5 TCR alone, 78% of $58^{\alpha-\beta-}$ cells were Vβ-11 positive and 37% bound tetramer, with an MFI of 1940 and 2277, respectively.

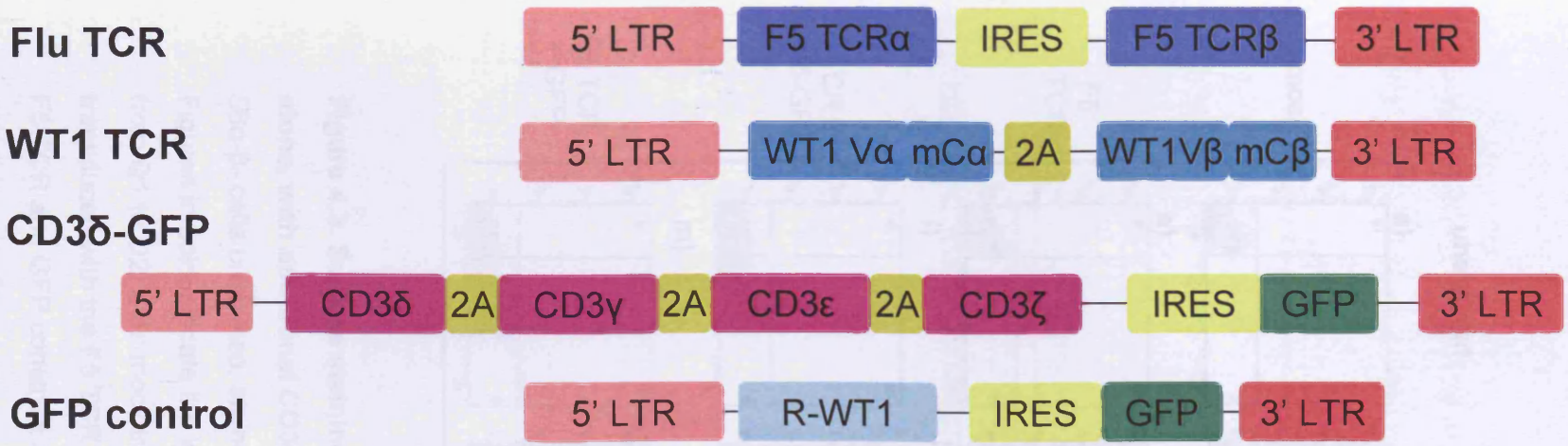

Figure 4.2 Schematic representation of retroviral vectors

The F5 TCR vector is based on Moloney murine leukaemia virus. The F5 TCR recognises an epitope from the influenza protein presented by H2Db and the α and β chains are linked by an IRES. The WT1 TCR is a myeloproliferative sarcoma virus based vector. The WT1 TCR recognises the pWT126 peptide from the human WT1 protein in the context of HLA-A*0201 Class I molecules. The constant domains of the α and β chains have been replaced with murine TCR constant (mC) domains, mutated to contain additional cysteine residues which form an additional disulphide bond between the TCR chains. V: variable region, c: constant region. This vector uses a viral 2A sequence to link the α and β chains, and has also been codon optimised. The CD3δ-GFP vector is a murine stem cell virus based vector, containing the CD3 chains, linked by 2A sequences, in the order δ,γ,ε then ζ. This vector is also linked to a green fluorescent protein (GFP) marker by an internal ribosome entry site (IRES). The GFP control vector uses the same long terminal repeat (LTR) as the WT1 TCR vector and contains the WT1 gene inserted in reverse orientation (such that this gene is not translated), followed by IRES GFP (not codon optimized).

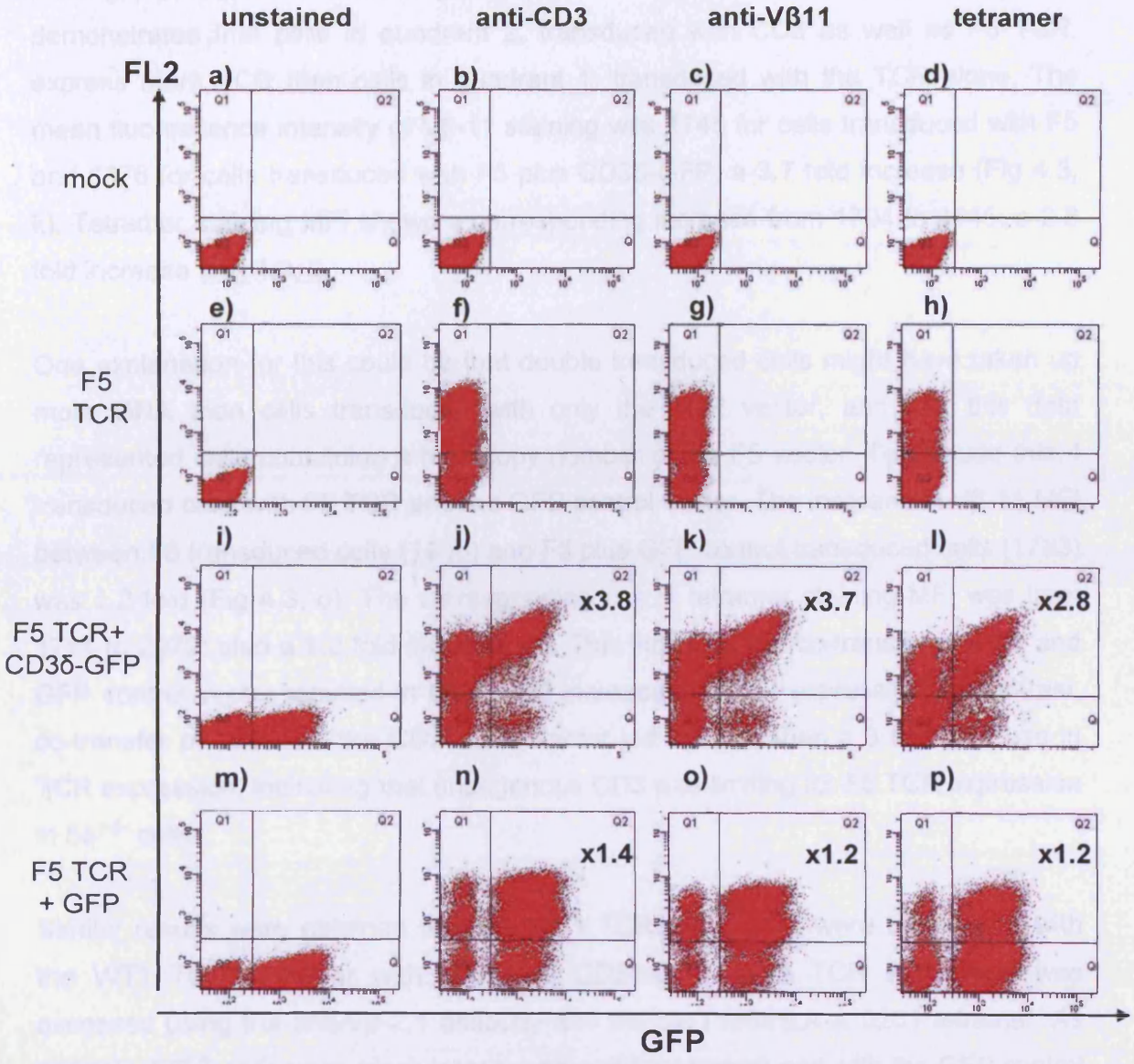

Figure 4.3. Surface staining of $58^{\alpha-\beta-}$ cells transduced with the F5 TCR alone, with additional CD3δ-GFP or with GFP control.

58α-β- cells unstained, stained with anti CD3-PE, anti Vβ11-PE and tetramer-PE. Figures in bold indicate the increase in antibody staining mean fluorescense intensity from Q1 to Q2. a-d): mock transduced; e-h): transduced with the F5 TCR ; i-l): transduced with the F5 TCR and CD3δ-GFP; m-p): 58α-β- cells transduced with F5 TCR and GFP construct as a control. Representative results from one experiment are shown (n=8)

$58^{\alpha-\beta-}$ cells transduced with F5 plus additional CD3 showed that the higher the CD3-GFP signal, the higher the surface expression of CD3 and Vβ-11, and tetramer binding was also increased (Fig 4.3, j-l). Within the same FACS plot it can be demonstrated that cells in quadrant 2, transduced with CD3 as well as F5 TCR, express more TCR than cells in quadrant 1, transduced with the TCR alone. The mean fluorescence intensity of Vβ-11 staining was 1745 for cells transduced with F5 and 6376 for cells transduced with F5 plus CD3δ-GFP; a 3.7 fold increase (Fig 4.3, k). Tetramer staining MFI shows a corresponding increase from 1794 to 4945: a 2.8 fold increase (Fig 4.3, l).

One explanation for this could be that double transduced cells might have taken up more DNA than cells transduced with only the TCR vector, and that this data represented cells containing a high copy number of the F5 vector. To exclude this, I transduced cells with F5 TCR and the GFP control vector. The increase in Vβ-11 MFI between F5 transduced cells (1438) and F5 plus GFP control transduced cells (1783) was 1.2 fold (Fig 4.3, o). The corresponding rise in tetramer staining MFI was from 1711 to 2079: also a 1.2 fold increase (p). This indicates the co-transfer of TCR and GFP control vector resulted in only small increases in TCR expression. In contrast, co-transfer of TCR and the CD3δ-GFP vector led to more than a 3 fold increase in TCR expression, indicating that endogenous CD3 was limiting for F5 TCR expression in $58^{\alpha-\beta-}$ cells.

Similar results were obtained with the WT1 TCR. $58^{\alpha-\beta-}$ cells were transduced with the WT1 TCR alone or with additional CD3δ-GFP. WT1 TCR expression was assessed using the anti-Vβ-2.1 antibody and the pWT126/HLA-A*0201 tetramer. As controls, $58^{\alpha-\beta-}$ cells were mock transduced and also transduced with the GFP control vector as above. Mock transduced $58^{\alpha-\beta-}$ cells did not express surface CD3, Vβ-2.1 or bind tetramer (Fig 4.4, a-d). After transduction with WT1 TCR alone, 79% of $58^{\alpha-\beta-}$ cells were Vβ-2.1 positive, but only 0.2% bound pWT126/HLA-A*0201 tetramer (Fig 4.4; g, h). $58^{\alpha-\beta-}$ cells transduced with WT1 TCR plus additional CD3 again showed that the higher the CD3δ-GFP signal, the higher the surface expression of CD3 and Vβ-2.1, and pWT126/HLA-A*0201 tetramer binding was also increased (Fig 4.4, j-l). The mean fluorescence intensity of Vβ-2.1 staining was 457 for cells transduced with WT1 TCR and 1715 for cells transduced with WT1 plus CD3: a 3.8 fold increase (Fig 4.4, k). WT1 tetramer MFI shows a corresponding increase from 160 to 361: a 2.2 fold increase (Fig 4.4, l). Hence, cells transduced with CD3 as well as WT1 TCR express more TCR than cells transduced with the WT1 TCR alone. The GFP control

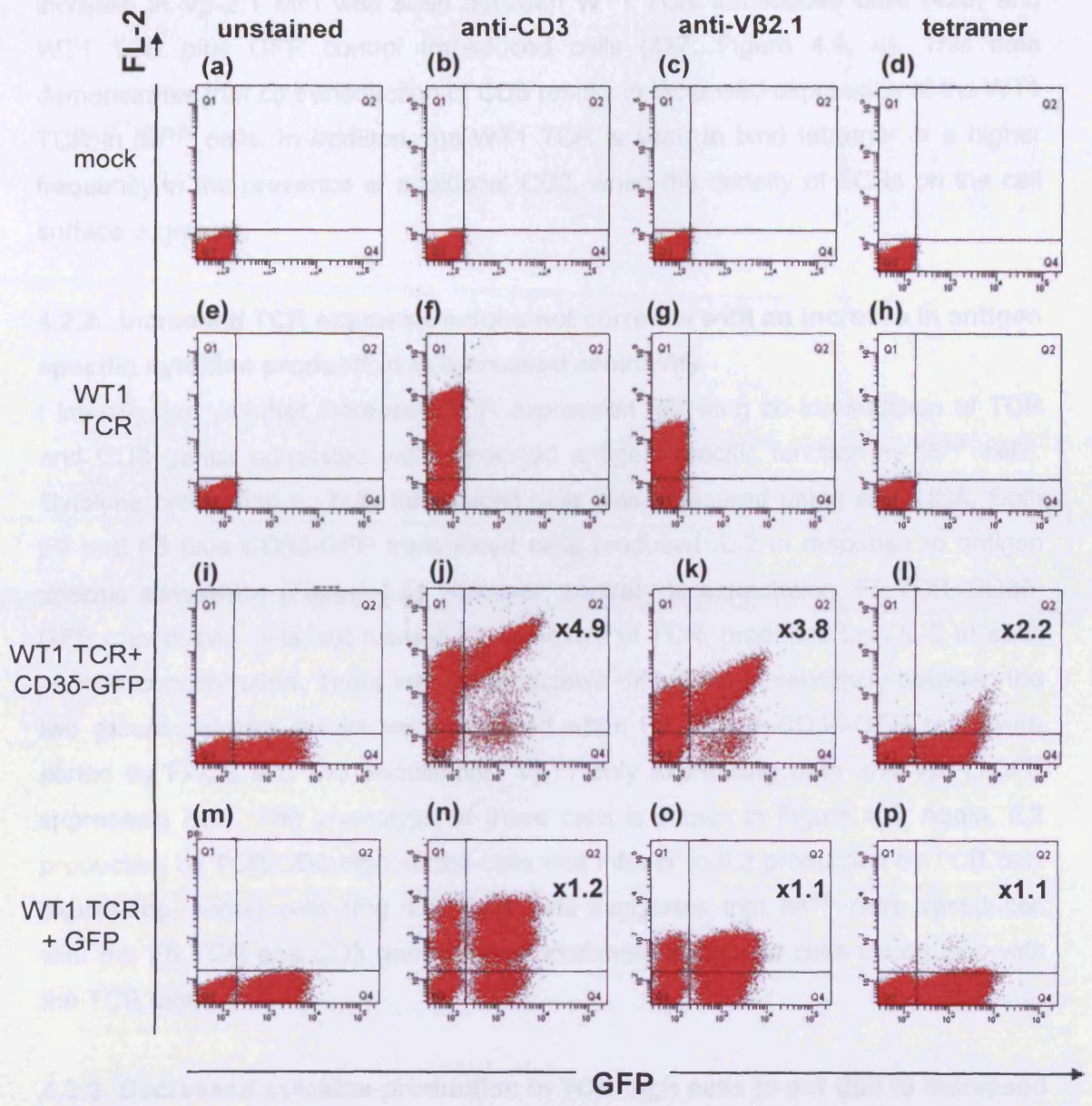

Figure 4.4. Surface staining of 58$^{\alpha-\beta-}$ cells transduced with the WT1 TCR alone or with additional CD3. 58α-β- cells unstained, stained with anti CD3-PE, anti Vβ2.1-PE and tetramer-PE. Figures in bold indicate the increase in antibody staining mean fluorescense intensity from Q1 to Q2. a-d): mock transduced; e-h): transduced with WT1 TCR ; i-l): transduced with the WT1 TCR and CD3δ-GFP; m-p): 58α-β- cells transduced with WT1 TCR and GFP construct as a control. Representative results from one experiment are shown (n=8)

again demonstrates that the increase in MFI is due to additional CD3: a 1.1 fold increase in Vβ-2.1 MFI was seen between WT1 TCR transduced cells (428) and WT1 TCR plus GFP control transduced cells (477; Figure 4.4, o). This data demonstrates that co transduction of CD3 results in increased expression of the WT1 TCR in $58^{\alpha-\beta-}$ cells. In addition, the WT1 TCR is seen to bind tetramer at a higher frequency in the presence of additional CD3, when the density of TCRs on the cell surface is greater.

4.2.2. Increased TCR expression does not correlate with an increase in antigen specific cytokine production or increased sensitivity

I investigated whether increased TCR expression following co-transduction of TCR and CD3 genes correlated with enhanced antigen specific function in $58^{\alpha-\beta-}$ cells. Cytokine production by bulk transduced cells was measured using an ELISA. Both F5 and F5 plus CD3δ-GFP transduced cells produced IL-2 in response to antigen specific stimulation (Figure 4.5). However, contrary to expectation, F5 TCR+CD3δ-GFP transduced cells, expressing higher levels of TCR, produced less IL-2 at each peptide concentration. There was no detectable difference in sensitivity between the two groups. Similar results were obtained when F5 TCR + CD3δ-GFP cells were sorted by FACS into two populations: Vβ11 only expressing cells and Vβ11+GFP expressing cells. The phenotype of these cells is shown in Figure 4.6. Again, IL2 production by TCR/CD3 high sorted cells was inferior to IL2 production by TCR only expressing, sorted cells (Fig 4.6). This data suggested that $58^{\alpha-\beta-}$ cells transduced with the F5 TCR and CD3 genes were functionally inferior to cells transduced with the TCR alone.

4.2.3 Decreased cytokine production in TCR high cells is not due to increased apoptosis.

I went on to explore the mechanism underlying the decreased cytokine production seen in $58^{\alpha-\beta-}$ cells expressing high CD3 and TCR levels. The signal delivered through the TCR plays an important role in determining T cell fates, such as activation, proliferation, anergy and apoptosis (207-209). Peripheral T cell death is required to control T cell expansion and to eliminate cells following the induction of an effective immune response (210). One mechanism by which this occurs is activation induced cell death (AICD), in which activation via the TCR results in apoptosis (209). The nature and intensity of TCR signalling are among the factors that determine the occurrence of AICD. I therefore investigated whether CD3/TCR high cells were being driven into apoptosis upon ligation of the TCR.

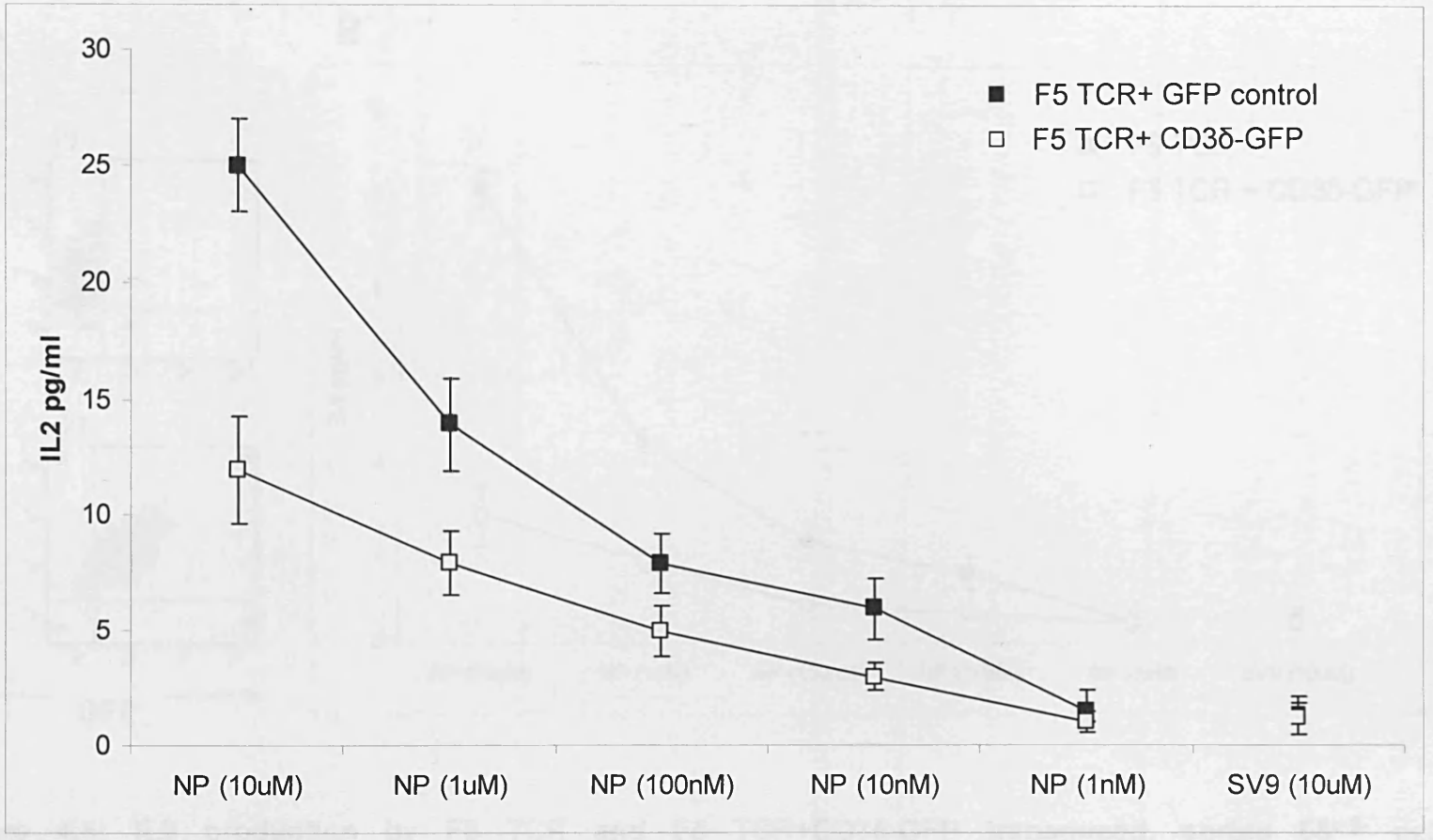

Figure 4.5: IL2 production by F5 TCR and F5 TCR+CD3δ-GFP transduced $58^{\alpha-\beta-}$ cells. Bulk transduced F5 TCR and F5 TCR+CD3δ-GFP cells were stimulated for 24 hours with RMA-S cells loaded with relevant (NP366) or irrelevant (pSV9) peptides. Tenfold dilutions of relevant peptide were used (10μM-1nM). Supernatant was harvested as assessed for IL2 production by ELISA. Shown is the mean IL2 concentration (pg/ml) +/- standard deviation of triplicate values. Representative data is shown from 5 repeat experiments. The phenotype of the cells used is shown in Figure 4.3

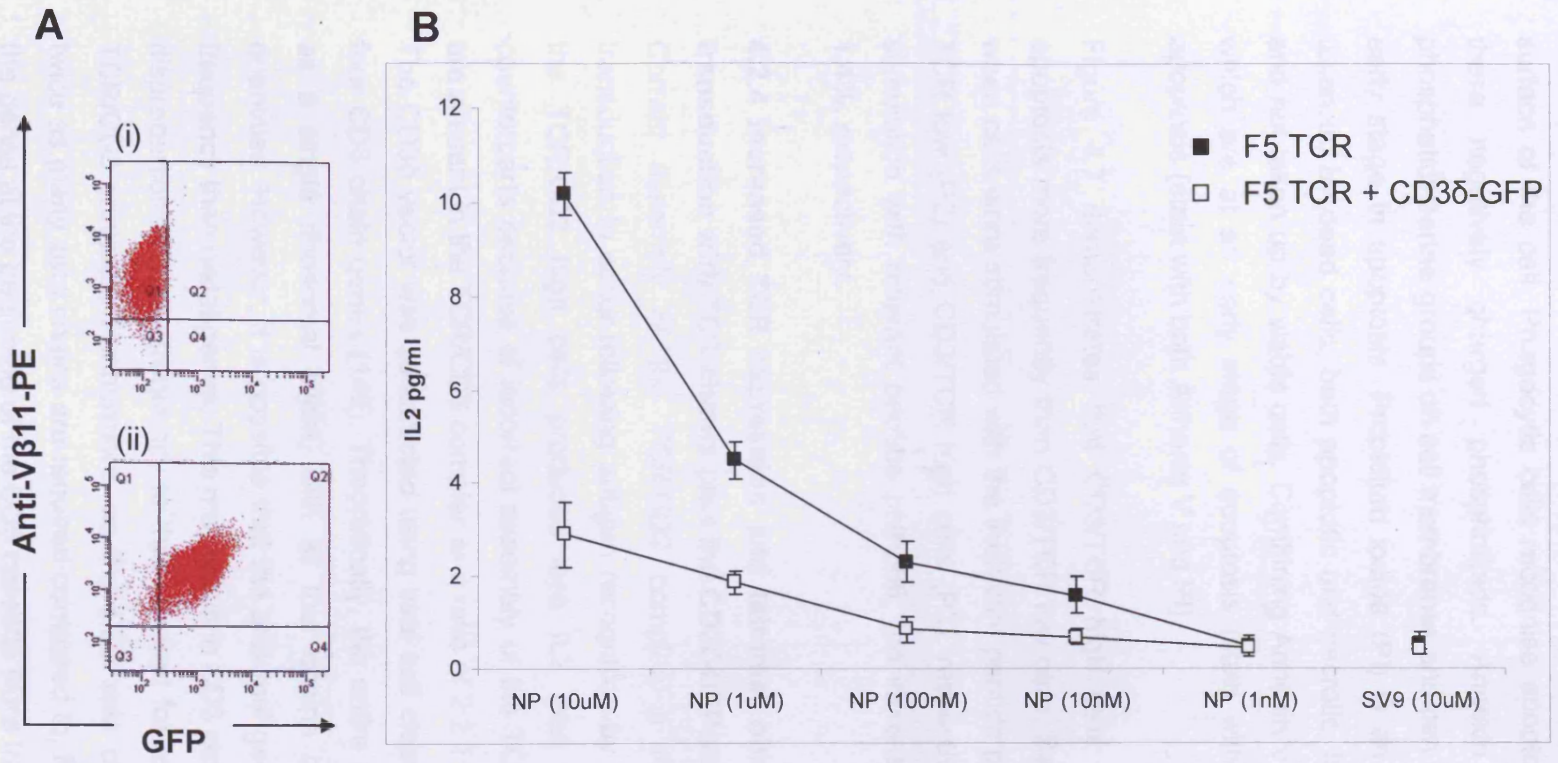

Figure 4.6: IL2 production by F5 TCR and F5 TCR+CD3δ-GFP transduced, sorted $58^{\alpha-\beta-}$ cells.
A: phenotype of sorted (i) F5 TCR and (ii) F5 TCR+CD3δ-GFP transduced $58^{\alpha-\beta-}$ cells stained with anti-Vβ11 antibody.

B: Sorted F5 TCR and F5 TCR+CD3δ-GFP transduced cells were stimulated for 24 hours with RMA-S cells loaded with relevant (NP366) or irrelevant (pSV9) peptides. Tenfold dilutions of relevant peptide were used (10μM-1nM). Supernatant was harvested as assessed for IL2 production by ELISA. Shown is the mean IL2 concentration (pg/ml) +/- standard deviation of triplicate values. Representative data is shown from 5 repeat experiments.

When cells undergo apoptosis, negatively charged phosphatidylserine groups which normally reside on the interior side of the cell membrane are transported to the outer surface of the cell. Phagocytic cells recognise apoptotic cells by the presence of these negatively charged phospholipids. Annexin V also binds to these phosphatidylserine groups on cell membranes and can be used to identify cells at an early stage in apoptosis. Propidium iodide (PI) is an intercalating agent which is taken up by dead cells, both apoptotic and necrotic, but is membrane impermeant and not taken up by viable cells. Combining Annexin V and PI staining reveals cells which are at an early stage of apoptosis (stain with Annexin V only) or in late apoptosis (stain with both Annexin V and PI).

Figure 4.7 demonstrates that CD3/TCR high cells were not being driven into apoptosis more frequently than CD3/TCR low cells. Background levels of apoptosis when cells were stimulated with the irrelevant peptide pSV9 were 1.2% and 2.0% for TCR low (P2) and CD3/TCR high cells (P3), respectively (Fig 4.7; b) i & ii). Upon stimulation with relevant peptide pNP366, the increase in apoptosis was 2% and 1.4%, respectively.

4.2.4 Increased TCR expression and tetramer binding is seen following co-transduction with TCR chains plus the CD3ζ-GFP vector

Correct assembly of the TCR/CD3 complex is required in order for signal transduction to occur following antigen recognition by the TCR. I hypothesized that the TCR/CD3 high cells produced less IL2 than their TCR only transduced counterparts because of incorrect assembly of the TCR/CD3 complex. CD3 chains are present in the TCR/CD3 complex at a ratio of 2:2:1:1 for ε,ζ,δ and γ, respectively. The CD3δ vector was constructed using viral self cleaving 2A sequences to link the four CD3 chain genes (149). Theoretically, the entire cassette should be translated as a single ribosomal event, with all four chains being produced in equimolar quantities. However, it is possible that the proximal genes are translated at a higher frequency than distal genes. This may result in CD3 zeta chains being translated at a disproportionately low frequency to that required for assembly of the fully functional TCR/CD3 complex. Furthermore, as the CD3 zeta chain exists as a homodimer, twice as many zeta chains are required compared to, for example the delta chain. If the genes at the beginning of the CD3 cassette were translated at a higher frequency than the ε and ζ genes at the end of the cassette, there would be a relative excess of δ and γ chains, which might favour the expression of functionally inferior TCR/CD3 complexes lacking the ζζ homodimer. The CD3 ζ chain plays a key role in

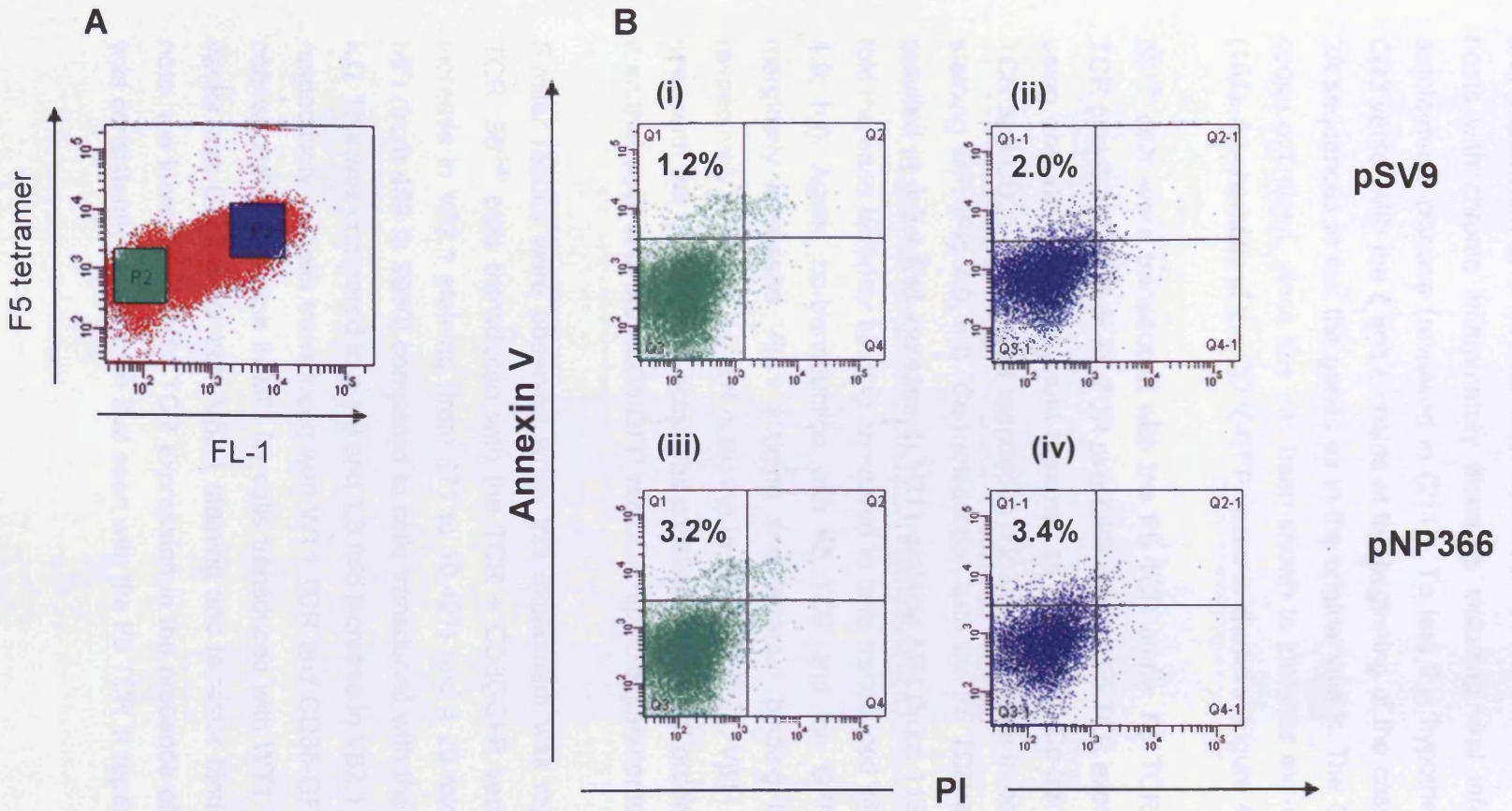

Figure 4.7: Annexin V staining of stimulated F5/CD3δ-GFP transduced $58^{\alpha-\beta-}$ cells

A: FACS plot demonstrating gating of P2: TCR low, GFP-ve cells; and P3: TCR high, CD3-GFP high cells.

B: Annexin V/PI staining of P2 and P3 after culture for 24 hours with RMA-S cells loaded with relevant (pNP366) and irrelevant (pSV9) peptides. Figures indicate the percentage of cells which stained with Annexin V only

receptor assembly, expression and signalling. Downregulation of ζ chain expression and impairment of T cell function has been demonstrated in T cells isolated from hosts with chronic inflammatory diseases including viral infections, cancers and autoimmune disease (reviewed in (211)). To test this hypothesis, I redesigned the CD3 vector with the ζ and ε chains at the beginning of the cassette, using the same 2A sequences to link the genes as in the original vector. The CD3 chains were also codon optimised, since this has been shown to increase expression of TCR genes (151). A schematic of the CD3ζ-GFP vector is shown in Figure 4.8.

$58^{\alpha-\beta-}$ cells were transduced with the F5 TCR alone, F5 TCR plus CD3δ-GFP, F5 TCR plus CD3ζ-GFP or F5 TCR plus GFP control. F5 TCR expression was assessed using anti-Vβ-11 antibody and tetramer as previously. Co-transduction with the F5 TCR and CD3δ-GFP vector resulted in a 2.3 fold increase in both Vβ11 and tetramer staining MFI (Fig 4.9; e,f). Co-transduction with the F5 TCR and CD3ζ-GFP vector resulted in a 5.4 fold increase in Vβ11 staining MFI (from 1199 to 6431) and a 4.3 fold increase tetramer binding compared to cells transduced with the TCR alone (Fig 4.9; h,i). Again, co-transduction with F5 TCR and the GFP control vector only marginally increased Vβ11 staining and tetramer binding by 1.1 and 1.2 fold, respectively (Fig 4.9; k,l). Of note, the increase in CD3, Vβ11 and tetramer binding seen with the CD3ζ-GFP vector was consistently several orders of magnitude higher than that seen with the CD3δ-GFP vector in repeat experiments.

Similar results were observed when this experiment was repeated with the WT1 TCR. $58^{\alpha-\beta-}$ cells transduced with the TCR + CD3ζ-GFP vector showed a 16 fold increase in Vβ2.1 staining (from 671 to 10,427) and a 20 fold increase in tetramer MFI (from 489 to 9930) compared to cells transduced with the TCR alone (Fig 4.10; k,l). This was compared to a 1.9 and 1.3 fold increase in Vβ2.1 and tetramer staining, respectively, in cells transduced with WT1 TCR and CD3δ-GFP (Fig 4.10; g,h). The corresponding increase in MFI for cells transduced with WT1 TCR and GFP control vector was 0.9 fold for both Vβ2.1 staining and tetramer binding (Fig 4.10; o,p). Of note, the increase in WT1 TCR expression in the presence of additional CD3ζ-GFP was consistently higher than that seen with the F5 TCR in repeat experiments.

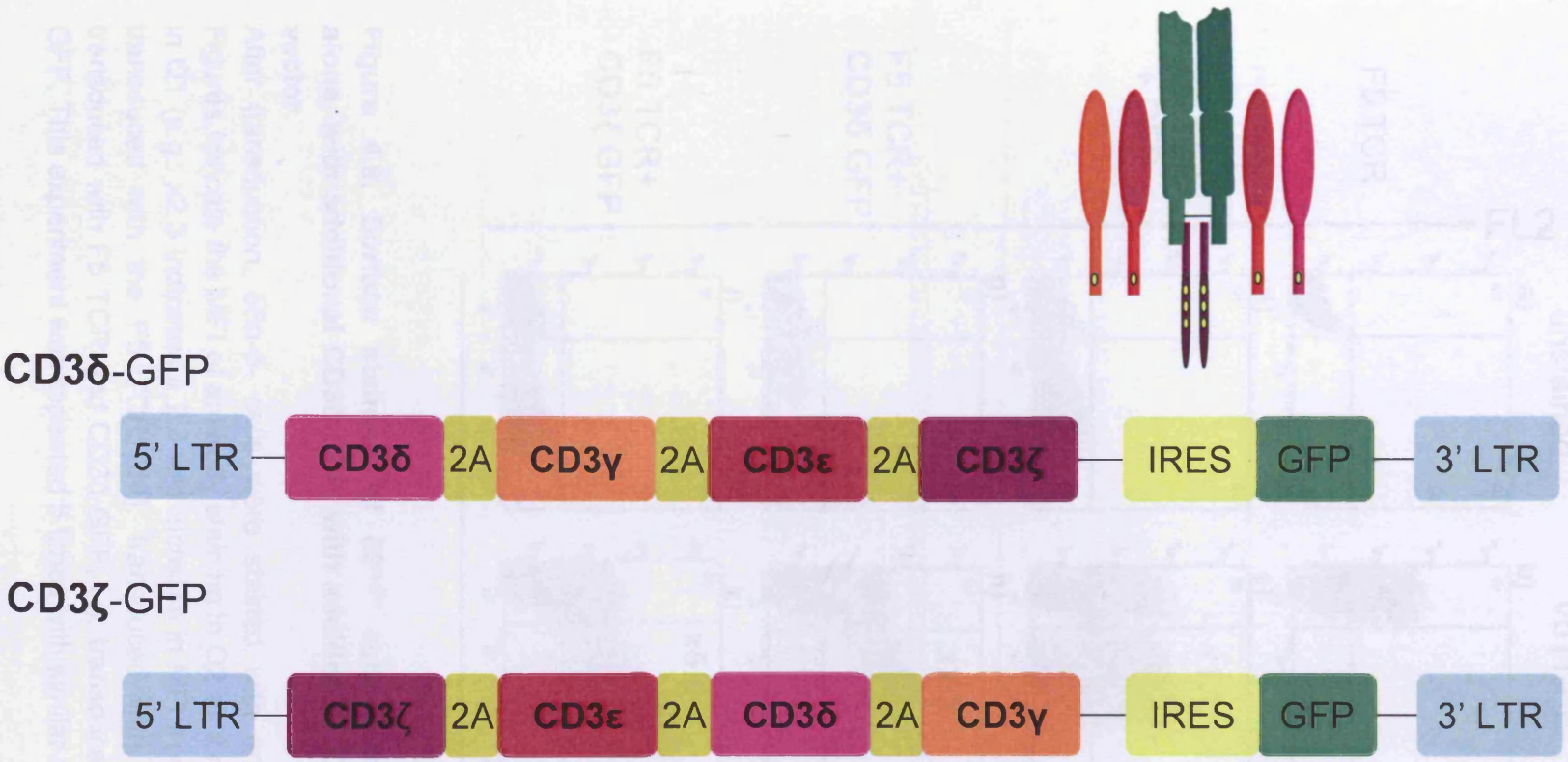

Figure 4.8: a new CD3 vector design

In the CD3ζ-GFP construct, the four CD3 chains are linked by the same 2A sequences as the CD3δ-GFP construct, but the order of the CD3 chains has been changed. The CD3δ-GFP construct is based on murine stem cell virus, whereas the CD3ζ-GFP vector uses the myoproliferative sarcoma virus LTR, and has also been codon optimized.

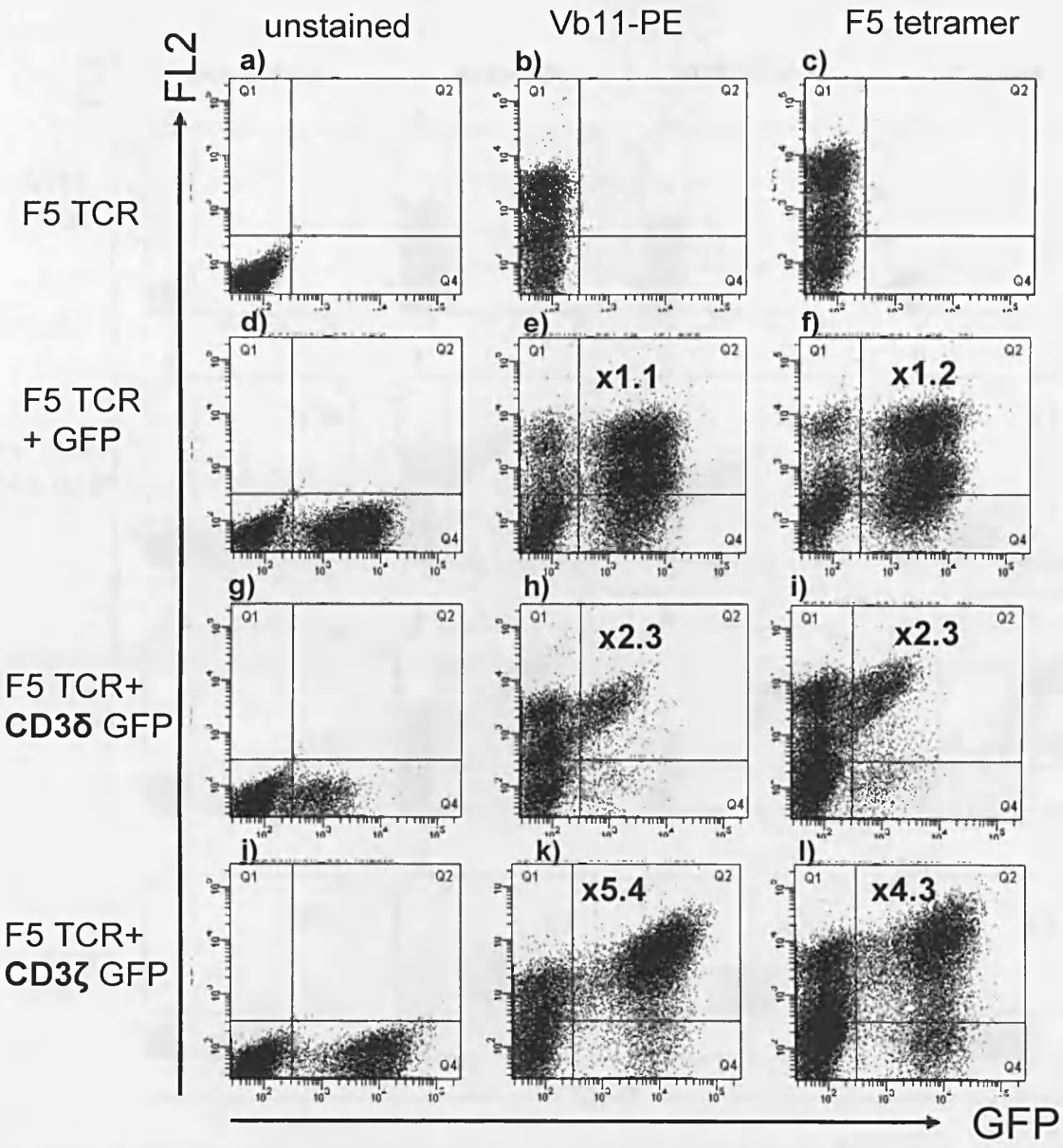

Figure 4.9. Surface staining of $58^{\alpha-\beta-}$ cells transduced with the F5 TCR alone, with additional CD3δ-GFP, with additional CD3ζ-GFP or with GFP control vector.

After transduction, 58α-β- cells were stained with anti Vβ11-PE and tetramer-PE. Figures indicate the MFI of antibody staining in Q2 as a multiple of the antibody staining in Q1 (e.g.: x2.3 indicates a 2.3 fold increase in MFI from Q1 to Q2). a-c): 58α-β- cells transduced with the F5 TCR; d-f): transduced with the F5 TCR and GFP; g-i): transduced with F5 TCR and CD3δ-GFP; j-l): transduced with the F5 TCR and CD3ζ-GFP. This experiment was repeated 5 times with similar results.

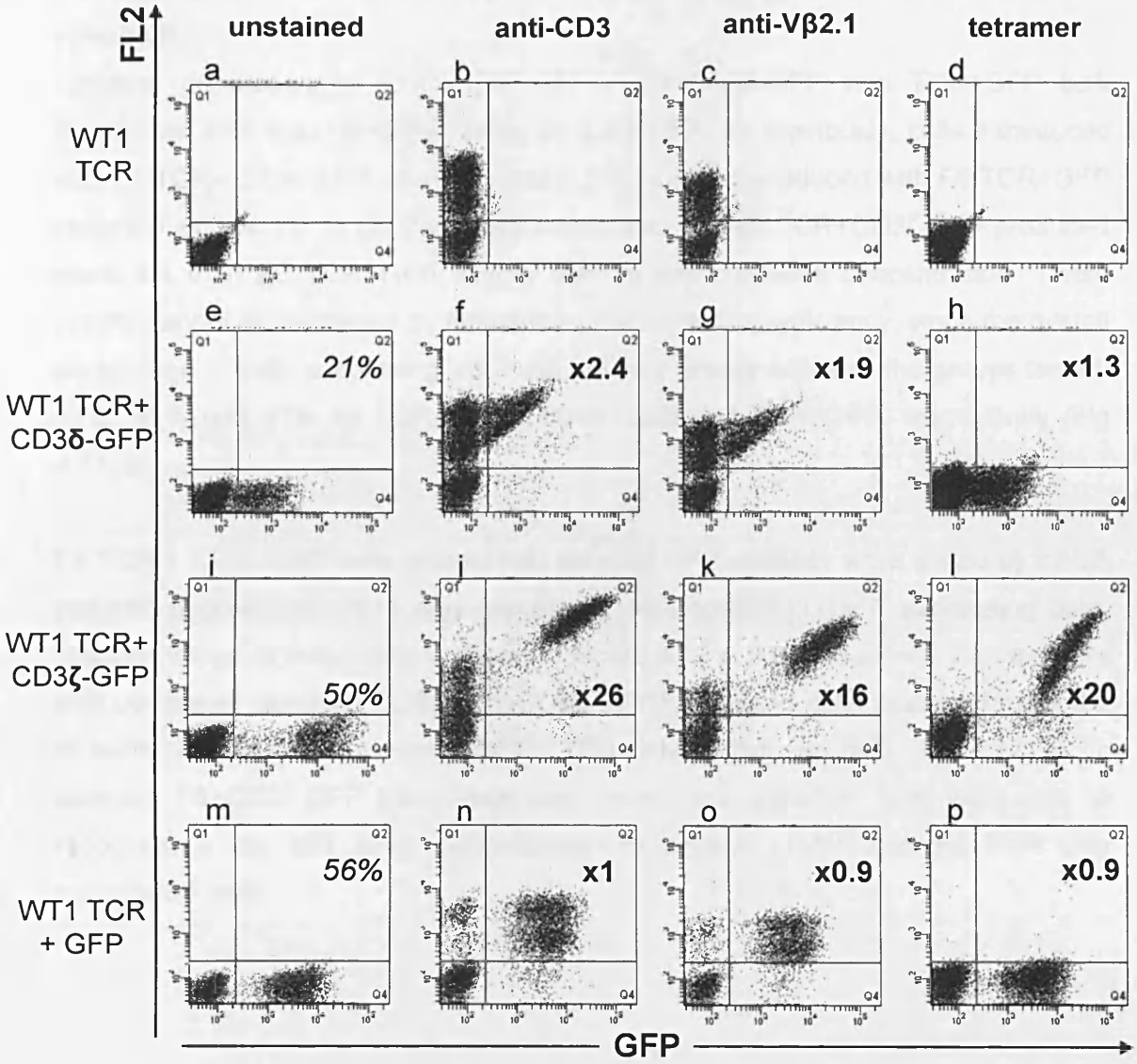

Figure 4.10. Surface staining of $58^{\alpha-\beta-}$ cells transduced with the WT1 TCR alone, with additional CD3δ-GFP, with additional CD3ζ-GFP or with GFP control vector.

After transduction, 58α-β- cells were stained with anti-CD3, anti Vβ2.1 and tetramer. Figures in italics indicate events in Q4, figures in bold indicate the MFI of antibody or tetramer staining in Q2 as a multiple of the MFI in Q1 a-d): 58α-β- cells transduced with the WT1 TCR; e-h): transduced with the WT1 TCR and CD3δ-GFP; i-l): transduced with WT1 TCR and CD3ζ-GFP; m-p): transduced with the WT1 TCR and GFP control. Representative results from 1 experiment are shown. This experiment was repeated 5 times with similar results.

4.2.5 Increased TCR expression and tetramer binding in TCR/CD3ζ-GFP transduced $58^{\alpha-\beta-}$ cells correlates with increased IL2 production and increased sensitivity

Cytokine production by TCR+CD3ζ-GFP, TCR+CD3δ-GFP and TCR+GFP bulk transduced cells was compared using an IL2 ELISA. As previously, cells transduced with F5 TCR+CD3δ-GFP produced less IL2 than cells transduced with F5 TCR+GFP control (Figure 4.11). In contrast, cells transduced with F5 TCR+CD3ζ-GFP produced more IL2 than F5 TCR+GFP control cells at every peptide concentration. These results cannot be explained by differences in transduction efficiency, since the overall percentage of cells expressing Vβ11 did not vary greatly between the groups tested: 61%, 57% and 57% for TCR+CD3ζ, TCR+CD3δ and TCR+GFP, respectively (Fig 4.11, b).

F5 TCR + CD3ζ-GFP cells stained with anti-Vβ11-PE antibody were sorted by FACS into two populations: Vβ11 only expressing cells and Vβ11+GFP expressing cells. The phenotype of these cells is shown in Figure 4.12 a. IL2 production by sorted cells was compared using an ELISA. F5+CD3ζ-GFP transduced cells produced more IL2 at each peptide concentration than F5 TCR only transduced cells (Fig 4.12, b). In addition, F5+CD3ζ-GFP transduced cells were more sensitive: they were able to recognise a ten fold lower concentration of peptide (1nM) than F5 TCR only transduced cells.

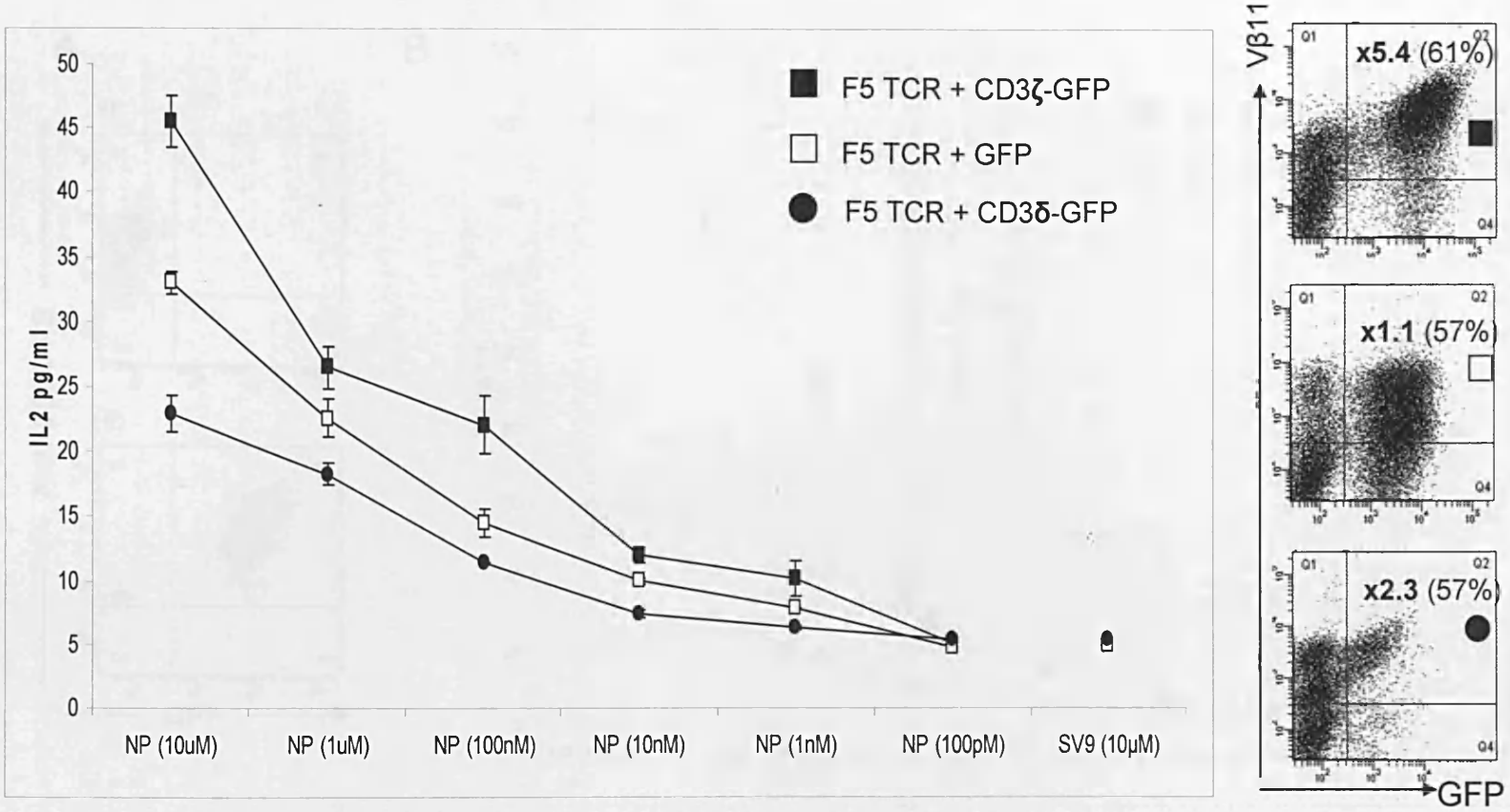

Figure 4.11: IL2 production by F5 TCR, F5 TCR+CD3δ-GFP and F5 TCR+CD3ζ-GFP transduced $58^{\alpha-\beta-}$ cells. A: Bulk transduced F5 TCR, F5 TCR+CD3δ-GFP and F5 TCR+CD3ζ-GFP cells were stimulated for 24 hours with RMA-S cells loaded with relevant (NP366) or irrelevant (pSV9) peptides. Tenfold dilutions of relevant peptide were used (10µM-1nM). Supernatant was harvested as assessed for IL2 production by ELISA. Shown is the mean IL2 concentration (pg/ml) +/- standard deviation of triplicate values. B: The phenotype of the cells used is shown. Representative data is shown from 3 repeat experiments

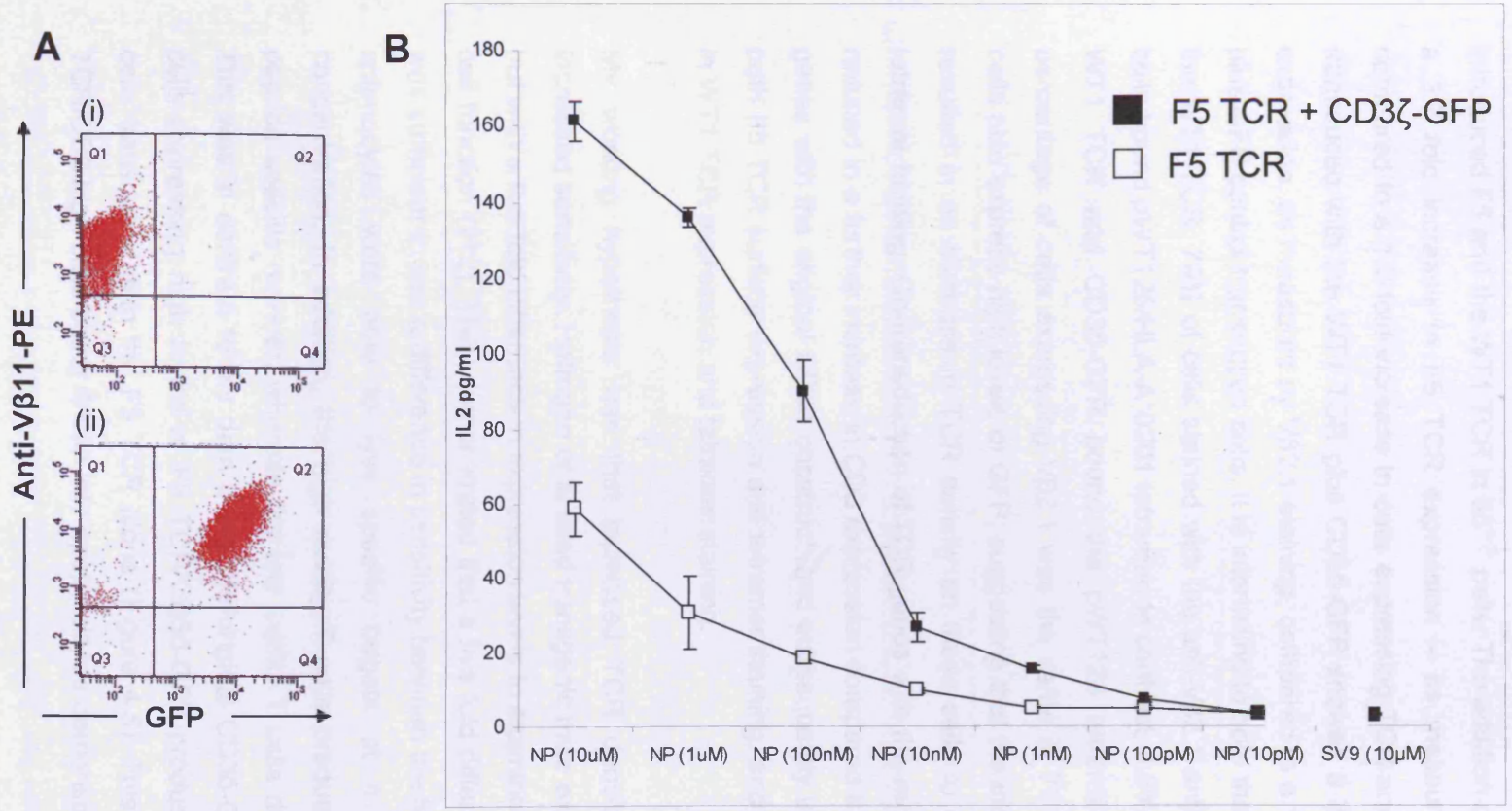

Figure 4.12: IL2 production by F5 TCR and F5 TCR+CD3ζ-GFP transduced, sorted $58^{\alpha-\beta-}$ cells. A: phenotype of sorted (i) F5 TCR and (ii) F5 TCR+CD3ζ-GFP transduced $58^{\alpha-\beta-}$ cells stained with anti-Vβ11 antibody.

B: Sorted F5 TCR and F5 TCR+CD3δ-GFP transduced cells were stimulated for 24 hours with RMA-S cells loaded with relevant (NP366) or irrelevant (pSV9) peptides. Tenfold dilutions of relevant peptide were used (10μM-10pM). Supernatant was harvested as assessed for IL2 production by ELISA. Shown is the mean IL2 concentration (pg/ml) +/- standard deviation of triplicate values. Representative data is shown from 3 repeat experiments.

4.3 Discussion

I have demonstrated that CD3 is a rate limiting step for the expression of both the introduced F5 and the WT1 TCR in $58^{\alpha-\beta-}$ cells. The addition of CD3δ-GFP resulted in a 3.7 fold increase in F5 TCR expression – as measured by Vβ11 staining - compared to a 1.2 fold increase in cells expressing TCR and the GFP control. Cells transduced with the WT1 TCR plus CD3δ-GFP showed a 3.8 fold increase in TCR expression, as measured by Vβ2.1 staining, compared to a 1.1 fold increase in TCR plus GFP control transduced cells. It is interesting to note that, after transduction with the WT1 TCR, 79% of cells stained with the anti-Vβ2.1 antibody, but only 0.2% of cells bound pWT126/HLA-A*0201 tetramer. In contrast, 4.5% of cells transduced with WT1 TCR and CD3δ-GFP bound the pWT126 tetramer, although the overall percentage of cells expressing Vβ2.1 was the same at 78%. The tetramer binding cells also express high levels of GFP, suggesting that the increased CD3 expression resulted in an increase in TCR density on these cells to a level which facilitated tetramer binding. Co-transduction of TCR genes with the modified CD3ζ-GFP vector resulted in a further increase in CD3 expression compared to co-transduction of TCR genes with the original CD3δ construct, and subsequently up to a 6 fold increase in both F5 TCR surface expression and tetramer staining, and up to a 16 fold increase in WT1 TCR expression and tetramer staining.

My working hypothesis was that increased TCR density would correlate with increased sensitivity. Hofmann *et al* used transgenic mice expressing the same TCR, but with a five fold difference in expression levels to examine the effect of avidity on T cell function (212). They demonstrated that a five fold difference in TCR expression was sufficient to see a difference in sensitivity between the two groups: the TCR high splenocytes were able to lyse specific targets at a ten-fold lower peptide concentration. In addition, the high avidity T cells produced IL-2 and IFN-γ in a peptide specific manner, whereas the low avidity T cells did not produce cytokine. This was in contrast to my data using the original CD3δ-GFP vector, where $58^{\alpha-\beta-}$ cells expressing high levels of F5 TCR/CD3δ-GFP produced less IL-2 than $58^{\alpha-\beta-}$ cells transduced with the F5 TCR alone (Figure 4.5). This was not a result of the TCR/CD3 high cells being driven into apoptosis, as demonstrated by Figure 4.5.

It is well documented that TCR/CD3 complexes can be expressed on the surface of T cells in the presence of ζ chain downregulation (213-215). The ζ chain plays a crucial role in signal transduction upon ligation of the TCR, and it has been demonstrated that T cells which have downregulated CD3 ζ chain expression are functionally

impaired. This phenomenon has been observed in T cells obtained from patients with chronic viral infections, cancers and autoimmune diseases (reviewed in 211). In conditions of chronic antigen exposure and chronic IFN-γ production, myeloid suppressor cells are thought to contribute to downregulation of the ζ chain through L arginine metabolism, as it has recently been shown that L arginine is required for expression of the ζ chain (216;217). I hypothesized that TCR/CD3δ-GFP transduced cells were functionally impaired due to incorrect TCR/CD3 assembly, resulting from CD3 ζ chains being translated at a disproportionately low frequency to that required for assembly of the fully functional TCR/CD3 complex. The new CD3ζ-GFP construct was designed to test this hypothesis, with the ζ chain at the front of the cassette. As with the CD3δ-GFP construct, co-transduction with the CD3ζ-GFP construct and TCR also resulted in increased TCR expression in $58^{\alpha-\beta-}$ cells. However, with the CD3ζ-GFP vector the increased TCR expression correlated with increased IL2 production as well as increased sensitivity. $58^{\alpha-\beta-}$ cells expressing high levels of F5 TCR/CD3 produced more IL2 at each peptide concentration than F5 TCR transduced cells, and also produced IL2 in response to a ten fold lower concentration of target antigen (Fig 4.12). This data is in keeping with Hoffman *et al*'s findings that increased TCR density correlates with increased sensitivity, and suggests that the CD3ζ-GFP vector resulted in the production of the 4 CD3 genes at a more favourable ratio than with the CD3δ-GFP construct, such that the TCR/CD3 complexes which were expressed on the cell surface were also functional.

It would be interesting to look at the ratio of the different CD3 chains in $58^{\alpha-\beta-}$ cells transduced with TCR or TCR plus CD3δ-GFP, to see if the CD3ζ chain is proportionately under-represented in the latter group. This could be achieved via intracellular staining and a comparison of the ratios of the MFI of the different chains. However, there is currently no anti-murine CD3-ζ antibody commercially available for intracellular staining. An alternative approach would be to use immunoprecipitation to assess the relative presence of the different ζ chains, the working hypothesis being that the ζ chain is under-expressed in the TCR/CD3δ-GFP high cells.

It was interesting to note that the increase in CD3 expression, and subsequently TCR expression, seen with the CD3ζ-GFP vector was consistently several orders of magnitude higher than that seen with the CD3δ-GFP vector. This is likely to be a result of the changes incorporated in the new CD3ζ-GFP construct design. Previous work in this laboratory has demonstrated that the pMP71 vector LTR used by the CD3ζ-GFP construct is more efficient than the murine stem cell virus LTR used by

the CD3δ-GFP cassette. A stronger LTR would result in increased transcription of the cassette. In addition, codon optimisation of the CD3ζ-GFP construct would be expected to result in increased translation of the CD3 genes.

Another consistent finding was that the effect of co-transduction with CD3ζ-GFP on TCR expression was more marked with the WT1 TCR than the F5 TCR. The retroviral vectors for these TCRs use different LTRs, which may affect the level of α and β chains produced. The WT1 TCR was codon optimised, as this has been shown to increase the expression of exogenous TCRs in T cells (151). In addition, whereas the F5 TCR uses an IRES motif to link the α and β chains, the WT1 TCR vector uses a viral 2A sequence to link the α and β chains, which is likely to further increase the availability of WT1 TCR chains. It is likely that, as a result of these vector modifications, there is a more pronounced excess of WT1 α and β chains than F5 TCR chains. Therefore, the provision of large amounts of additional CD3ζ-GFP subsequently results in a higher level of WT1 TCR expression, as there are a greater number of WT1 TCR chains available to form TCR/CD3 complexes.

Chapter 5: CD3 is rate limiting for the expression of a TCR introduced into primary T cells by retroviral gene transfer

5.1 Introduction

In the previous chapter it was established that the expression level of TCRs introduced into $58^{\alpha-\beta-}$ cells by retroviral transduction can be increased several fold by co-transduction with CD3 molecules.

The situation with respect to the transduction of primary T cells is complicated by the presence of an endogenous TCR. Following TCR gene transfer, the introduced TCR will be in competition with the endogenous TCR for CD3 molecules (Figure 5.1). Introduced TCRs are often co-expressed with the endogenous TCR, which may reduce the density of introduced TCRs and hence affect their functional avidity. I hypothesized that the endogenous TCR and CD3 chains would be present in roughly equimolar quantities, and that there would not be a large excess of endogenous α and β TCR chains. Therefore, I would expect that the provision of additional CD3 would not increase endogenous TCR expression on the cell surface. However, production of the α and β chains of the exogenous TCR is under the control of the retroviral promoter, so I would expect that there will be a relative excess of exogenous TCR chains. The working hypothesis was that provision of additional CD3 molecules would therefore result in increased expression of the exogenous TCR. Furthermore, that increased TCR expression density would correlate with increased antigen specific cytokine production or sensitivity.

The aim of this chapter was to explore whether the co-transfer of TCR genes together with the genes encoding the γ,δ,ε and ζ chains of the CD3 complex can augment TCR expression. I have investigated the effect of additional CD3 on both endogenous TCR expression, as well as on the expression of an exogenous TCR introduced by retroviral gene transfer.

5.2 Results

5.2.1 Additional CD3 can increase endogenous TCR expression in primary T cells.

Activated C57BL/6 splenocytes were mock transduced, transduced with the CD3δ-GFP construct or the GFP control vector. Cells were stained with anti-CD3 and anti-TCR antibodies to monitor TCR expression levels. The MFI of anti-CD3 antibody

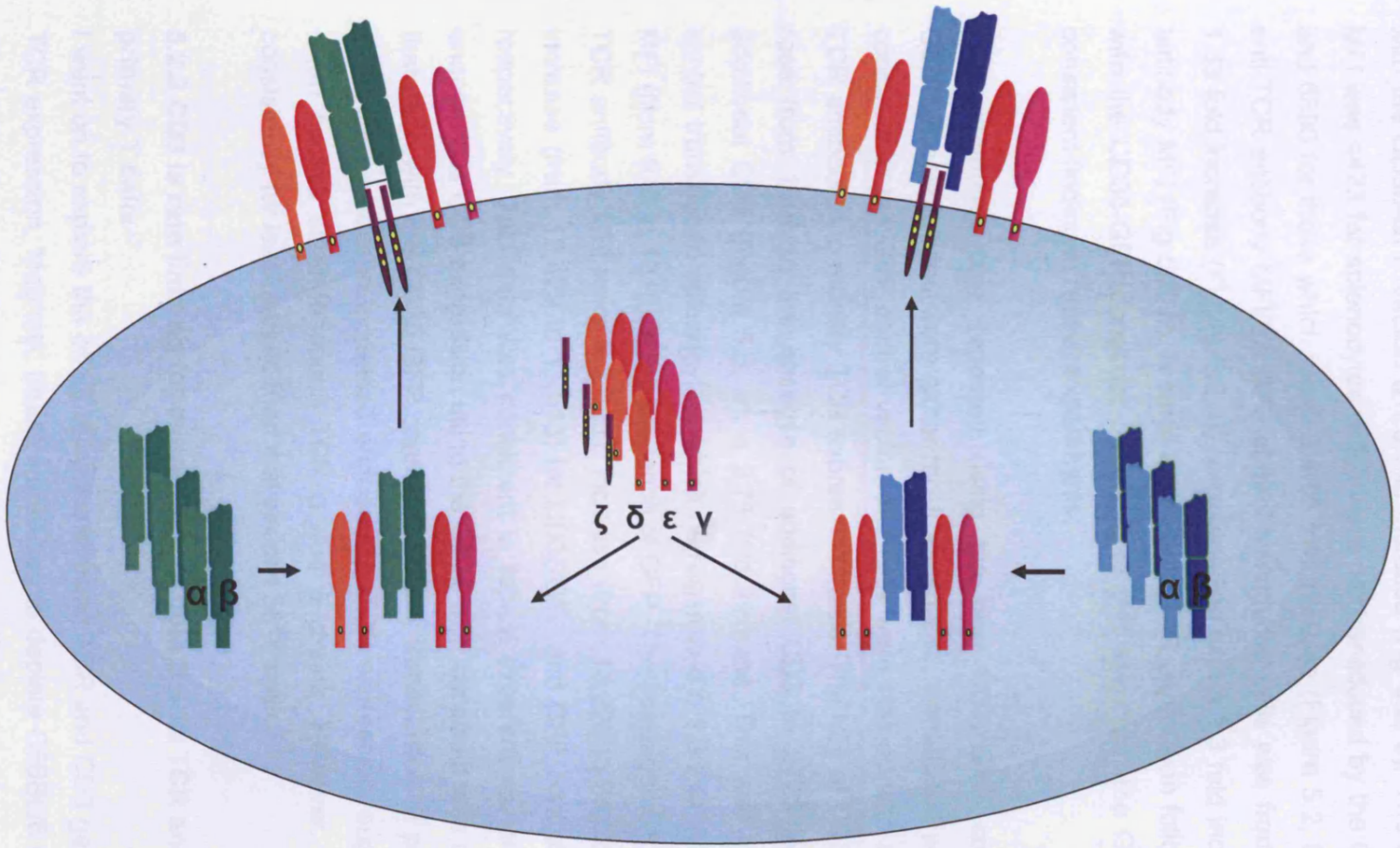

Figure 5.1: Competition between endogenous and exogenous TCRs for CD3 chains for surface expression TCR α and β chains must form a complex with the ζ,δ,ε and γ CD3 chains in order for the TCR to be expressed on the cell surface. Following retroviral TCR gene transfer, there is competition between the endogenous TCR (in green) and the exogenous TCR (in blue) for CD3. A lack of available CD3 chains may reduce the density of expression of the exogenous TCR, which could reduce its functional avidity.

staining was 747 for untransduced splenocytes and 1140 for splenocytes transduced with the CD3δ-GFP vector: a 1.3 fold increase (Fig 5.2, e). The anti-TCR antibody MFI was 4423 for splenocytes which were not transduced by the CD3δ-GFP vector, and 6890 for those which were: a 1.56 fold increase (Figure 5.2, f). The increase in anti-TCR antibody MFI for GFP control transduced cells was from 3238 to 4321: a 1.33 fold increase (Figure 5.2, i), corresponding with a 1.3 fold increase in anti-CD3 antibody MFI (Fig 5.2, h). A small increase in TCR expression following transduction with the CD3δ-GFP construct compared to that seen with the GFP control was a consistent finding in repeat experiments.

The experiment was repeated using the new CD3ζ-GFP construct: activated C57BL/6 splenocytes were either mock transduced, transduced with the CD3ζ-GFP construct or the GFP control vector, and cells were stained with anti-CD3 and anti-TCR antibodies to monitor TCR expression levels. The MFI of CD3 antibody staining rose from 1095 in the absence of additional CD3 to 2726 in the presence of additional CD3 (Figure 5.3, e): a 2.75 fold increase. This was in contrast to GFP control transduced splenocytes, which demonstrated a 1.3 fold increase in anti-CD3 MFI (from 976 to 1315) in the presence of GFP. The corresponding increases in anti-TCR antibody MFI were a 2.6 fold increase (from 13,222 to 34,522) and a 1.25 fold increase (from 12,429 to 15,513) for CD3ζ-GFP and GFP control transduced cells, respectively. This data was consistent in repeat experiments, and the increase in endogenous CD3 expression using the CD3ζ-GFP construct was always higher than that seen with the CD3δ-GFP construct. Hence, transduction of primary T cells with CD3ζ-GFP results in increased endogenous TCR expression, suggesting that there is an excess of endogenous TCR α and β chains. However, the increase was consistently far less marked than that seen in $58^{\alpha-\beta-}$ cells.

5.2.2 CD3 is rate limiting for expression of both the F5 TCR and the WT1 TCR in primary T cells

I went on to explore the effect of co-transducing TCR and CD3 genes on exogenous TCR expression. Magnetic beads were used to deplete C56BL/6 splenocytes of Vβ-11 expressing cells. Splenocytes were then activated and transduced with the F5 TCR alone, with additional CD3ζ-GFP or with the GFP control construct. Cells were stained with anti-CD3, anti-Vβ11 antibodies and tetramer to monitor expression of the exogenous TCR. Endogenous Vβ-11 expression and tetramer staining were minimal in mock transduced, Vβ-11 depleted T cells: 1.1% and 0.1% respectively. After transduction with the F5 TCR alone, Vβ-11 expression and tetramer staining

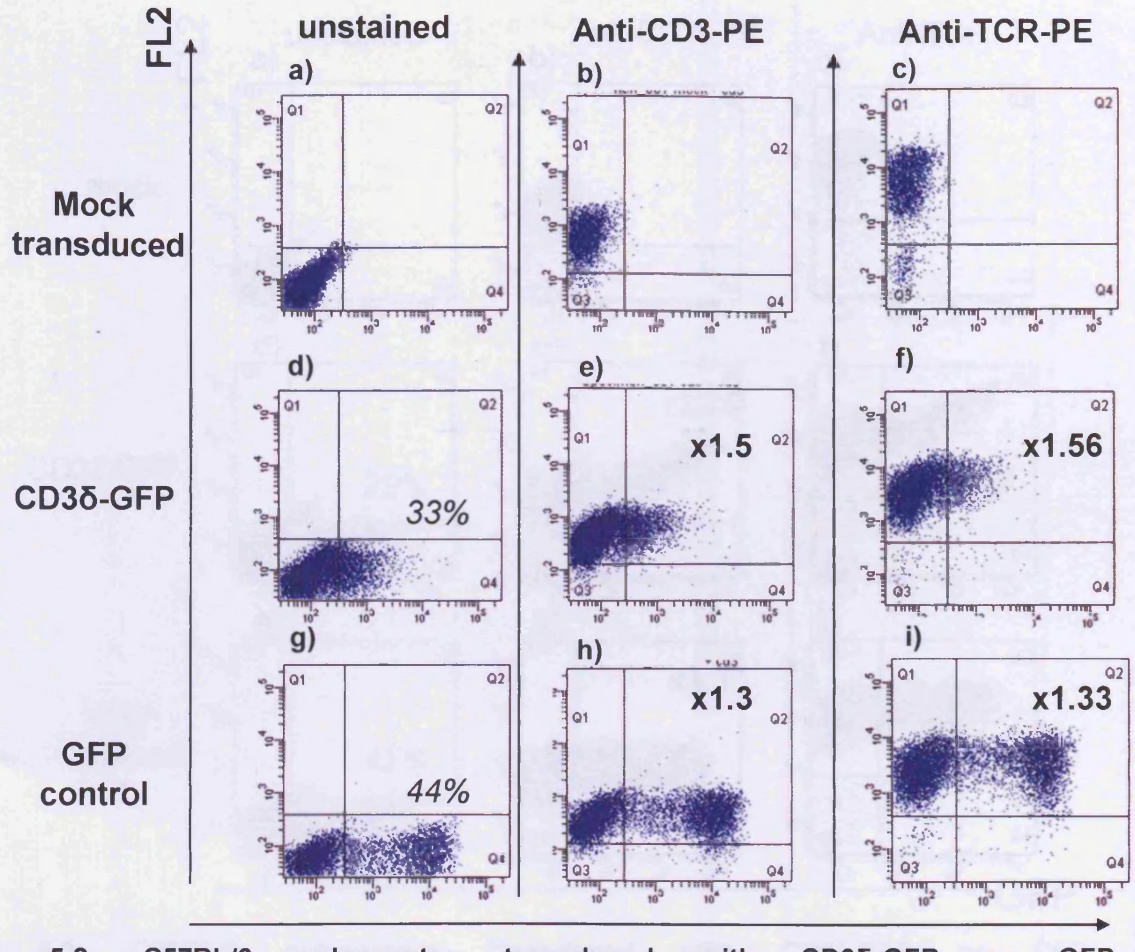

Figure 5.2: C57BL/6 splenocytes transduced with CD3δ-GFP or GFP control vector After transduction, C57BL/6 splenocytes were stained with anti-CD3-PE antibody or an antibody directed against the TCR β chain constant region (anti-TCR-PE). Figures in italics indicate the transduction efficiency/percentage of cells in Q4. Figures in bold indicate the increase in antibody staining mean fluorescense intensity from Q1 to Q2. a-c: mock transduced; d-f: transduced with CD3δ-GFP; g-i: transduced with GFP control vector. Representative results from 1 of 5 repeat experiments are shown.

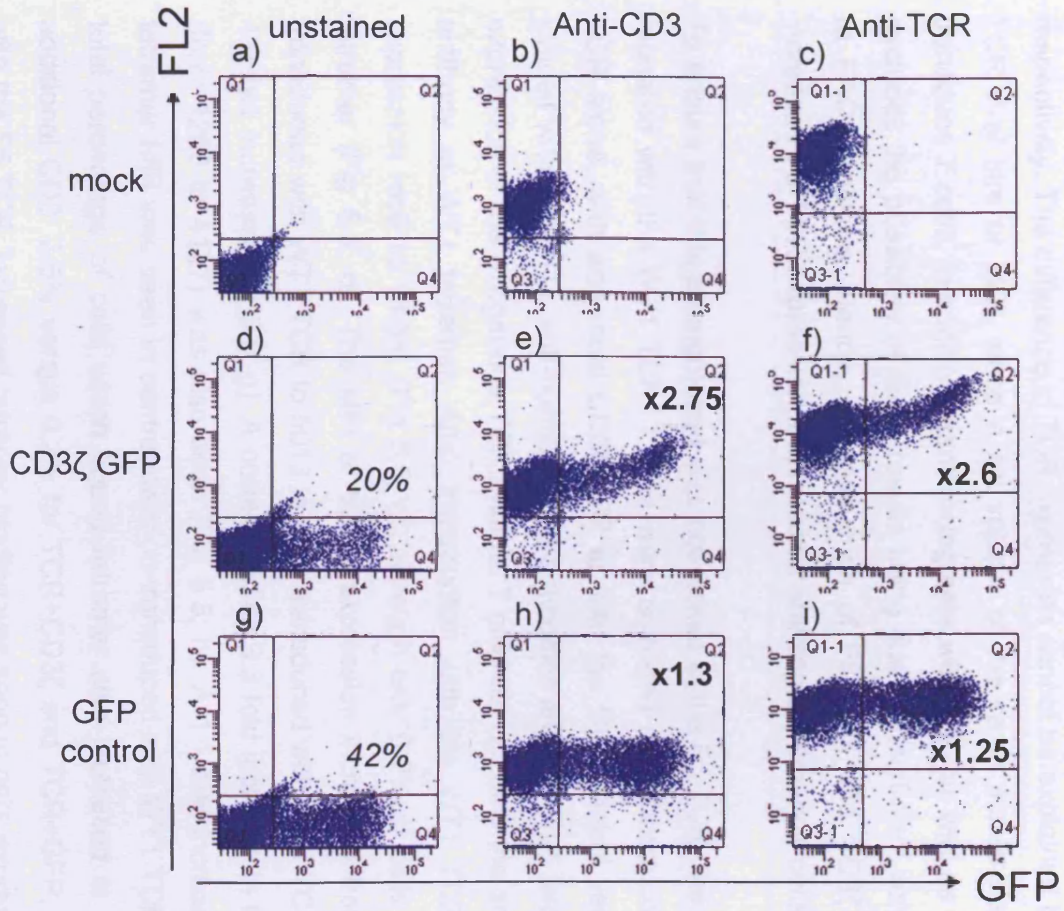

Figure 5.3: C57BL/6 splenocytes transduced with CD3ζ-GFP or GFP control vector After transduction, C57BL/6 splenocytes were stained with anti-CD3-PE antibody or an antibody directed against the TCR β chain constant region (anti TCR). Figures in italics indicate the transduction efficiency/percentage of cells in Q4. Figures in bold indicate the increase in antibody staining mean fluorescense intensity from Q1 to Q2. a-c: mock transduced; d-f: transduced with CD3ζ-GFP; g-i: transduced with GFP control vector. Representative results from 1 of 5 repeat experiments are shown.

rose to 15% and 1.3%, respectively (Fig 5.4; c,d). The MFI of Vβ-11 expression increased from 3973 to 13945 in cells transduced with F5 TCR and F5 TCR + CD3ζ-GFP: a 3.5 fold increase, accompanied by a 9.5 fold increase in tetramer staining (from 1343 to 12820) (Fig 5.4; g,h). The corresponding increase in Vβ11 and tetramer MFI seen in control cells co-transduced with F5 and GFP was 1.1 fold (Fig 5.4; k,l). The total percentage of cells which bound tetramer also increased in the presence of additional CD3: 3.7% versus 1.2% for TCR+CD3ζ and TCR+GFP, respectively. The difference in TCR expression cannot be explained by differences in TCR viral titre or MOI, since a set volume of the same supernatant was used to transduce T cells. In addition, transducing cells with TCR as well as the GFP control excludes the possibility of these results being due to insufficient compensation prior to FACS analysis. Hence, cotransduction of F5 TCR and CD3ζ-GFP results in increased F5 TCR expression and tetramer staining in primary T cells

To ensure that this phenomenon was not limited to the F5 TCR, the experiment was repeated with the WT1 TCR. T cells were activated and transduced with the WT1 TCR alone, with additional CD3ζ-GFP or with the GFP control vector. Cells were stained with anti-CD3, anti-human Vβ2.1 antibodies and pWT126 tetramer to monitor expression of the exogenous TCR. Murine T cells did not bind the anti-human Vβ2.1 antibody or WT1 tetramer. After transduction with the WT1 TCR alone, Vβ-2.1 expression rose to 18.3% (Fig 5.5; c), although only 0.2% of cells bound pWT126 tetramer (Fig 5.5; d). The MFI of Vβ2.1 expression increased from 1019 in cells transduced with WT1 TCR to 5013 in cells transduced with WT1 TCR+CD3ζ-GFP: a 4.9 fold increase (Fig 5.5; g). A corresponding 3.3 fold increase in tetramer staining (from 1260 to 4127) was also seen (Fig 5.5; h). A 1.1 fold increase in Vβ2.1 and tetramer MFI was seen in control cells co-transduced with WT1 TCR and GFP. The total percentage of cells which bound tetramer also increased in the presence of additional CD3: 2.5% versus 0.2% for TCR+CD3ζ and TCR+GFP, respectively. As with the F5 TCR, increased tetramer binding was seen in cells expressing the highest levels of CD3ζ-GFP.

5.2.3 Primary T cells transduced with TCR plus CD3ζ-GFP display increased antigen specific cytokine production and increased sensitivity

IL2 and IFNγ production by WT1 TCR+CD3ζ-GFP and WT1 TCR+GFP bulk transduced splenocytes was compared using an ELISA. The phenotype of the cells used is shown in Figure 5.5. Cells transduced with WT1 TCR+CD3ζ-GFP produced

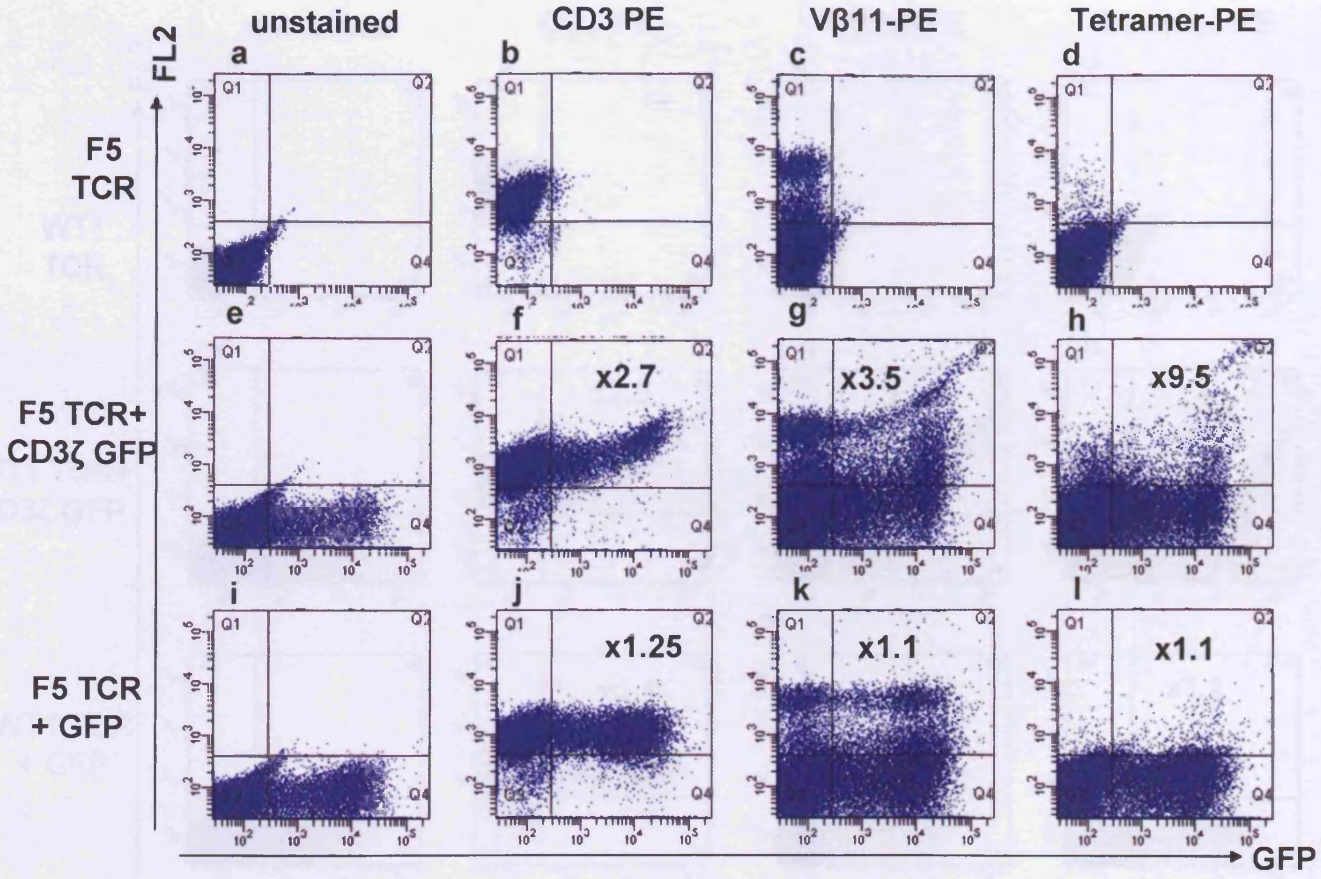

Figure 5.4: Surface staining of Vβ11 depleted C57BL/6 splenocytes transduced with the F5 TCR alone, F5 TCR plus CD3ζ-GFP or F5 TCR plus GFP control. C57BL/6 splenocytes were Vβ11 depleted prior to activation. Following transduction, cells were stained with anti-CD8α-APC and anti-CD3-PE, anti Vβ11-PE or tetramer-PE to assess F5 TCR expression. Shown are gated CD8+ cells. a-d): transduced with F5 TCR; e-h): transduced with F5 TCR + CD3ζ-GFP; i-l): transduced with F5 TCR and GFP control. Figures in bold indicate the increase in mean fluorescence intensity between Q1 and Q2. Representative data from 1 of 5 repeat experiments is shown.

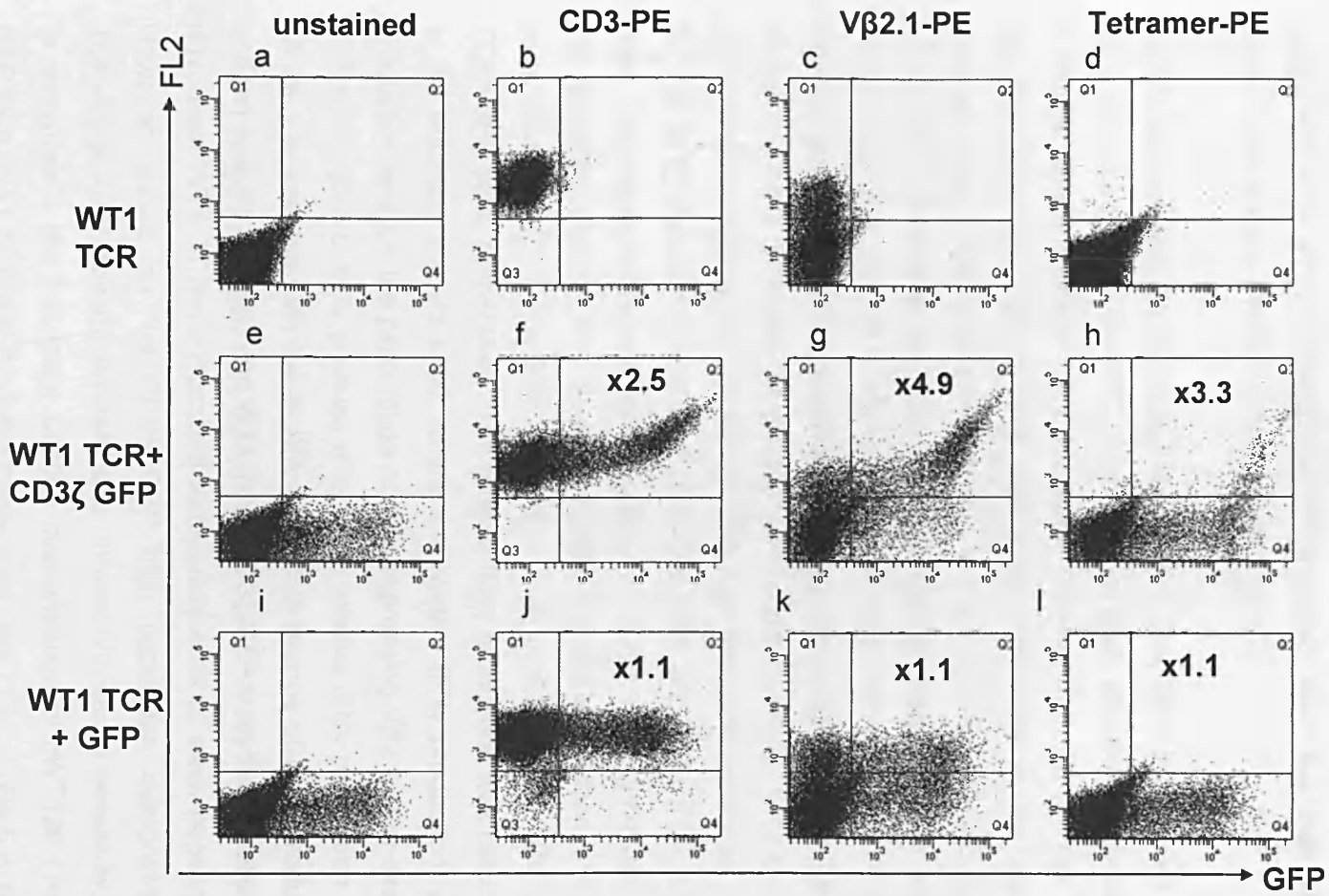

Figure 5.5: Surface staining of C56BL/6 splenocytes transduced with the WT1 TCR alone, WT1 TCR plus CD3ζ-GFP or WT1 TCR plus GFP control. Following transduction, cells were stained with anti-CD8α-APC and anti-CD3-PE, anti Vβ2.1-PE or WT1 tetramer-PE to assess WT1 TCR expression. Shown are gated CD8+ cells. Figures in bold indicate the increase in mean fluorescence intensity between Q1 and Q2. a-d): transduced with WT1 TCR; e-h): transduced with WT1 TCR + CD3ζ-GFP; i-l): transduced with WT1 TCR and GFP control. Representative data from 1 of 5 repeat experiments is shown.

more IFNγ than WT1 TCR+GFP control cells at each peptide concentration (Fig 5.6). These results cannot be explained by differences in transduction efficiency, since the overall percentage of cells expressing Vβ2.1 was similar in the two groups: 19.5% and 18% for WT1 TCR+CD3ζ and WT1 TCR+GFP, respectively. Similarly, cells transduced with WT1 TCR+CD3ζ-GFP produced more IL2 than WT1 TCR+GFP control cells at each peptide concentration (Fig 5.7).

ELISAs consistently demonstrated that TCR+CD3ζ-GFP transduced cells produced more cytokine than TCR+GFP control cells at each peptide concentration, but failed to clearly reveal a difference in sensitivity between the two groups. I hypothesized that this was due having used bulk transduced cells in the assays, and that comparing the TCR/CD3ζ-GFP high cells to the TCR/GFP cells would demonstrate a difference. I therefore went on to perform intracellular cytokine staining on WT1 TCR+CD3ζ-GFP and WT1 TCR+GFP transduced cells. Due to TCR down-regulation following antigen specific stimulation, I was unable to gate on Vβ2.1 expressing cells. However, I have previously shown that GFP correlates with TCR expression in CD3ζ-GFP transduced cells, so I gated on FITC high cells and compared IFNγ production by the GFP high cells in WT1 TCR+CD3ζ-GFP and WT1 TCR+GFP transduced cells. The gating strategy is shown in Figure 5.8 (a). Flow cytometric analysis of the two groups of cells on the day prior to setting up the stimulation revealed that Vβ2.1 expression was 97% and 53% in the GFP high populations of TCR+CD3ζ-GFP and TCR+GFP cells, respectively. The relative IFNγ production was calculated according to the equation x = y/z x100, where x = relative IFNγ production, y = actual IFNγ production and z = the percentage of cells expressing Vβ2.1. (For example, if 10% of TCR+GFP control cells produce IFNγ, then relative IFNγ production = 10/53 x100 = 18.9%.) In keeping with the ELISA data, both groups of cells produced IFNγ in an antigen specific manner, and WT1 TCR+CD3ζ-GFP transduced cells produced more IFNγ than WT1 TCR+GFP control transduced cells at each peptide concentration. However, gating on the CD8+ GFP high population demonstrated that WT1 TCR+CD3ζ-GFP transduced cells also showed increased sensitivity, and were able to recognise a ten fold lower peptide concentration of pWT126 (1nM) than CD8+, GFP high, WT1 TCR+GFP control transduced cells (10nM) (Fig 5.8; b).

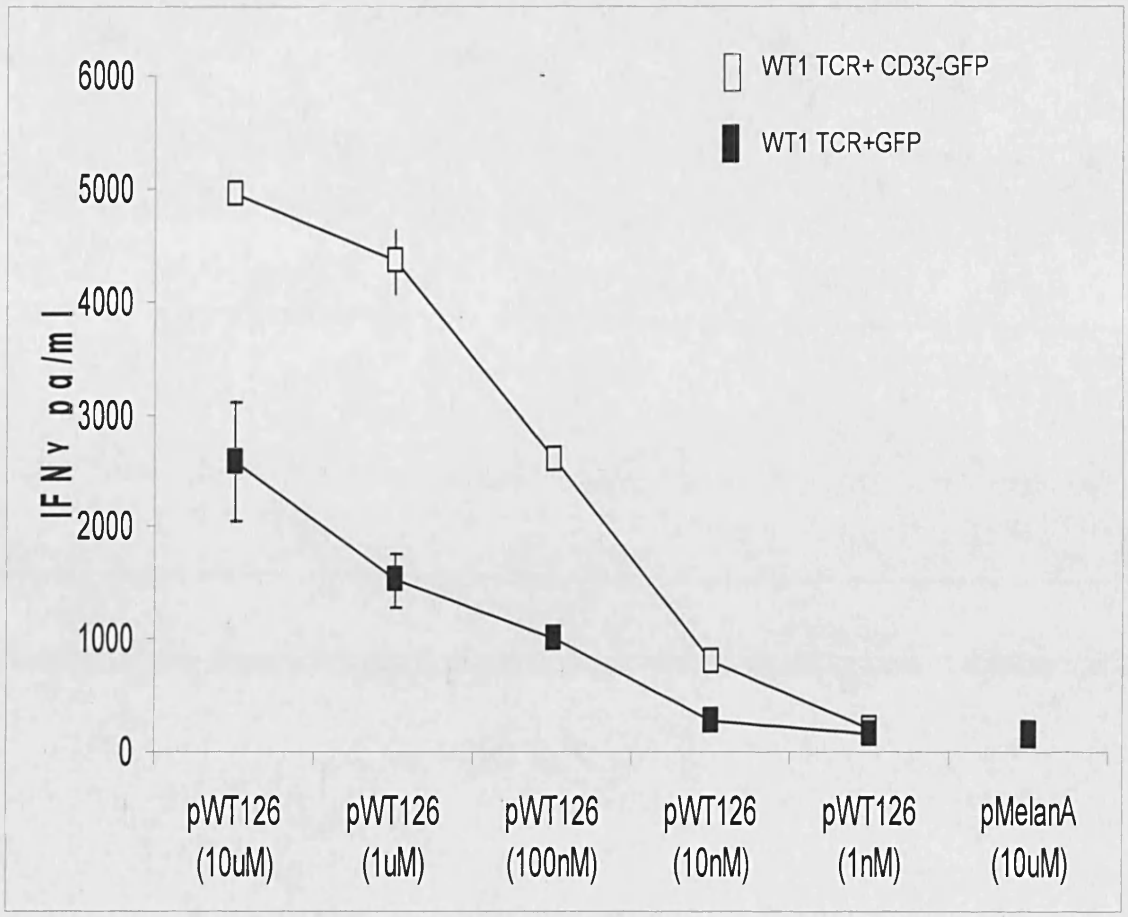

Figure 5.6: IFNγ production by WT1 TCR + CD3ζ-GFP and WT1 TCR + GFP control transduced cells. WT1 TCR + CD3ζ-GFP and WT1 TCR + GFP control transduced cells were stimulated for 24 hours with T2 cells loaded with relevant (pWT126) or irrelevant (pMelanA) peptides. Tenfold dilutions of relevant peptide were used (10μM-1nM). Supernatant was harvested and assessed for IFNγ production by ELISA. Shown is the mean IFNγ concentration (pg/ml) +/- standard deviation of triplicate values. Representative data is shown from 1 of 3 repeat experiments

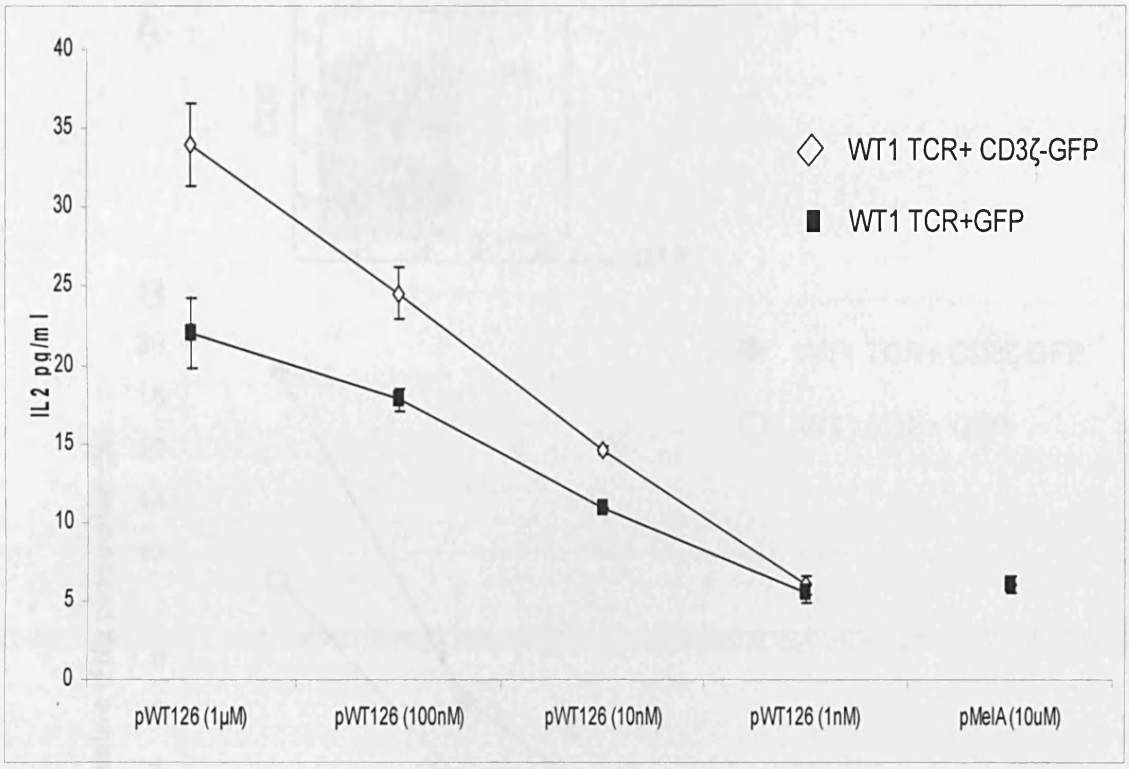

Figure 5.7: IL2 production by WT1 TCR + CD3ζ-GFP and WT1 TCR + GFP control transduced cells. WT1 TCR + CD3ζ-GFP and WT1 TCR + GFP control transduced cells were stimulated for 24 hours with T2 cells loaded with relevant (pWT126) or irrelevant (pMelanA) peptides. Tenfold dilutions of relevant peptide were used (10μM-1nM). Supernatant was harvested and assessed for IL2 production by ELISA. Shown is the mean IL2 concentration (pg/ml) +/- standard deviation of triplicate values. Representative data is shown from 1 experiment (n=3)

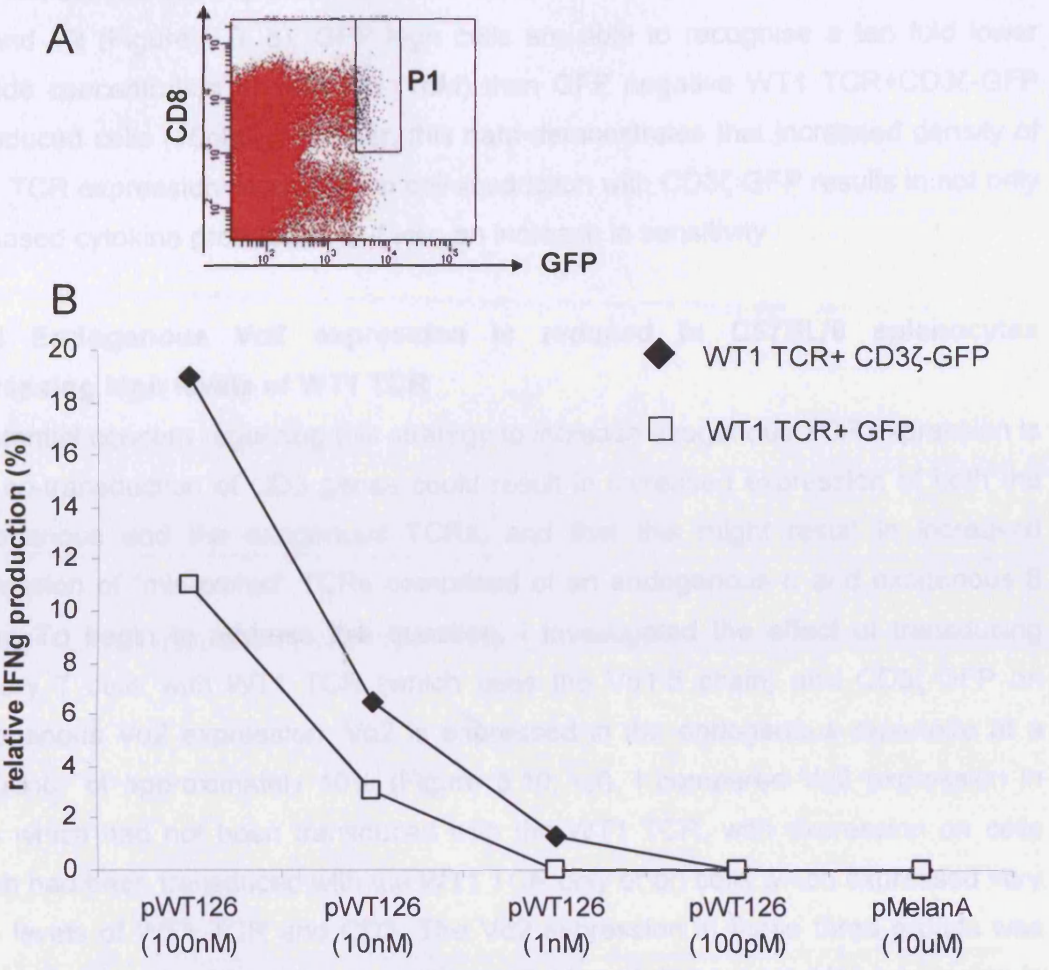

Figure 5.8: IFNγ production by WT1 TCR/CD3ζ-GFP and WT1 TCR/GFP transduced cells

Intracellular IFN- detection was performed on 1 x 106 T cells stimulated with 5 x 105 T2 cells loaded with either relevant (pWT126) or irrelevant (pMelanA) peptide. Tenfold dilutions of relevant peptide were used (1μM-100pM). After 2 hours, Brefeldin A was added to block cytokine secretion, and after a further 12 hours staining was performed. Cells were stained with anti-CD8-APC antibody, then fixed, permeabilized and stained with IFN-γ PE. A: of the CD8 positive lymphocytes, the GFP high population was selected (P1) for analysis of IFNγ production. B: IFNγ production by P1 in WT1+CD3ζ-GFP and WT1+GFP transduced cells. Due to differences in Vβ2.1 expression in the two groups analysed, IFNγ production was calculated according to the equation x = y/z x100, where x = relative IFNγ production, y = actual IFNγ production and z = %Vβ2.1 expression. Representative results from 1 of 3 repeat experiments are shown.

Gating on GFP negative (P1) and GFP high (P2) cells within the WT1 TCR+CD3ζ-GFP transduced cell population also demonstrates a difference in sensitivity between P1 and P2 (Figure 5.9; b): GFP high cells are able to recognise a ten fold lower peptide concentration of pWT126 (1nM) than GFP negative WT1 TCR+CD3ζ-GFP transduced cells (10nM). Together, this data demonstrates that increased density of WT1 TCR expression secondary to cotransduction with CD3ζ-GFP results in not only increased cytokine production, but also an increase in sensitivity.

5.2.4 Endogenous Vα2 expression is reduced in C57BL/6 splenocytes expressing high levels of WT1 TCR

A potential concern regarding this strategy to increase exogenous TCR expression is that co-transduction of CD3 genes could result in increased expression of both the endogenous and the exogenous TCRs, and that this might result in increased expression of "mis-paired" TCRs comprised of an endogenous α and exogenous β chain. To begin to address this question, I investigated the effect of transducing primary T cells with WT1 TCR (which uses the Vα1.5 chain) and CD3ζ-GFP on endogenous Vα2 expression. Vα2 is expressed in the endogenous repertoire at a frequency of approximately 10% (Figure 5.10; c,f). I compared Vα2 expression in cells which had not been transduced with the WT1 TCR, with expression on cells which had been transduced with the WT1 TCR only or on cells which expressed very high levels of WT1 TCR and CD3. The Vα2 expression in these three groups was 10.4%, 6.4% and 1.2%, respectively (Figure 5.11; c-e). In contrast, Vα2 expression in WT1 TCR and WT1 TCR plus GFP control expressing cells was almost unchanged at 5.8% and 5.7%, respectively (Fig 5.11; g,h). This data demonstrates that cells which expressed high levels of the WT1 TCR and CD3 expressed very low levels of Vα2, which does not support the hypothesis that there is increased endogenous TCR expression and subsequent mispairing in these cells.

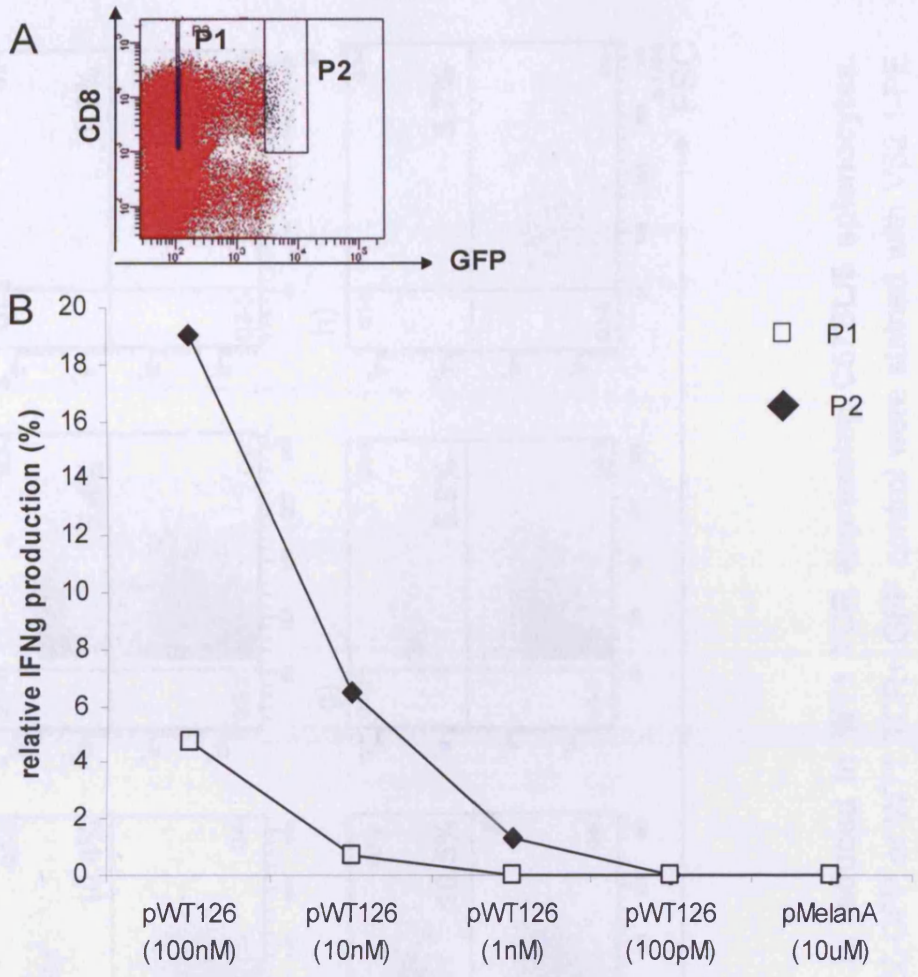

Figure 5.9: IFNγ production by GFP high and GFP negative WT1 TCR/CD3ζ-GFP transduced T cells

Intracellular IFNγ detection was performed using 1 x 106 T cells stimulated with 5 x 105 T2 cells loaded with either relevant (pWT126) or irrelevant (pMelanA) peptide. Tenfold dilutions of relevant peptide were used (1μM-100pM). After 2 hours, Brefeldin A was added to block cytokine secretion, and after a further 12 hours staining was performed. Cells were stained with anti-CD8-APC antibody, then fixed, permeabilized and stained with IFN- PE. A: of the CD8 positive lymphocytes, a GFP negative population (P1) and a a GFP high population (P2) were selected for analysis of IFNγ production. B: IFNγ production by WT1+CD3ζ-GFP transduced cells in P1 and P2. Due to differences in Vβ2.1 expression in the two groups analysed, IFNγ production was calculated according to the equation x = y/z x100, where x = relative IFNγ production, y = actual IFNγ production and z = %Vβ2.1 expression. Representative results from 1 of 3 repeat experiments are shown.

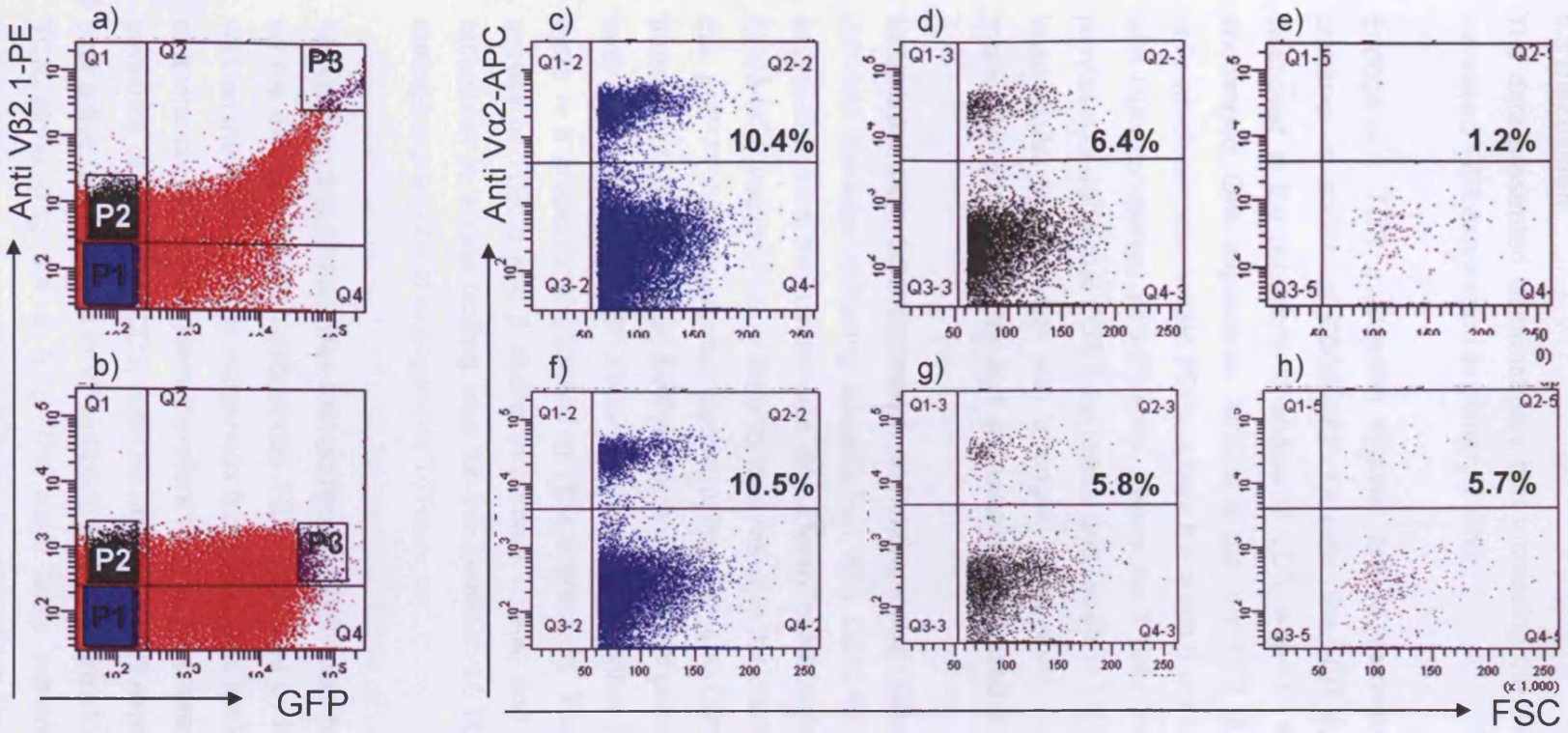

Figure 5.10: Endogenous Vα2 expression is reduced in WT1 TCR expressing C57BL/6 splenocytes. Splenocytes transduced with WT1 TCR+ CD3ζ-GFP or WT1 TCR+ GFP control were stained with Vβ2.1-PE and anti-Vα2-APC antibodies. The frequency of Vα2 chain expression was assessed in P1: cells which did not express the WT1 TCR (c and f); P2: cells which were WT1 TCR positive and GFP negative (d and g) and P3: the top 0.5% of GFP expressing cells, which also expressed the WT1TCR (e and h).

5.3 Discussion

The data presented demonstrates that increasing the availability of CD3 results in increased TCR expression in primary T cells.

Endogenous TCR expression showed only a modest 1.5 fold increase in the presence of additional CD3δ-GFP. Of note, the TCR dull population was seen to disappear in the presence of additional CD3, whereas the TCR high population is unchanged. One explanation for this is that the TCR dull population represents T cells which express 'weak' TCRs, where the α and β chains do not fold and associate with CD3 complexes as efficiently. Hence, for T cells expressing 'weak' TCRs, the provision of additional CD3 is beneficial and results in increased TCR expression to a level similar to that seen with 'stronger' TCRs which fold and associate with CD3 molecules more efficiently, and are naturally expressed at a higher density.

In contrast, both CD3 expression and endogenous TCR expression showed up to a 2.5 fold increase following transduction with CD3ζ-GFP. The level of CD3/GFP expression from this vector was consistently much higher than that seen with the CD3δ-GFP vector. This is likely to be a result of the combined effects of changing to the pMP71 LTR and also codon optimising the CD3ζ-GFP vector. In T cells transduced with the CD3ζ-GFP vector, the TCR dull population again disappears, as seen with the CD3δ-GFP vector. However, with further increases in CD3 provision, there is a proportional increase in TCR expression. This suggests that there is an excess of TCR α and β chains in primary T cells, and that the availability of CD3 molecules is a rate limiting step for the formation of TCR/CD3 complexes and for surface expression of endogenous TCRs.

My working hypothesis was that additional CD3 would have a more profound effect on the expression of an exogenous TCR introduced by retroviral gene transfer. This was on the basis that the exogenous α and β chains are expressed under the control of a viral promoter, and were therefore likely to be present in excess. Indeed, in the presence of additional CD3, both F5 and WT1 TCR expression consistently showed over a five fold increase in Vβ antibody MFI compared to T cells transduced with the TCR alone (Fig 5.4 & 5.5). The most likely explanation for the data is that endogenous and exogenous TCRs are in competition for CD3 for surface expression, and that increasing the availability of CD3 molecules allows a greater number of exogenous TCR chains to form TCR/CD3 complexes and be expressed on the cell surface. Figure 5.5 shows a 2.5 fold increase in CD3 expression in cells transduced

with CD3ζ-GFP. However, the increase in exogenous Vβ2.1 expression is greater at 4.9 fold. This is indirect evidence that the additional CD3 is exerting a proportionately greater effect on exogenous WT1 TCR expression compared to endogenous TCR expression.

It was interesting to note that cells transduced with either the WT1 TCR alone or the WT1 TCR + GFP control, which could be stained with the Vβ2.1 antibody, did not stain with the WT1 tetramer. One explanation for this could be that the introduced Vβ2.1 chain is mispairing with the endogenous α chain, and that the mixed dimer does not bind tetramer. However, it may also relate to the tetramer used, since cells transduced with the WT1 TCR or TCR + GFP consistently produced IL2 and IFNγ in an antigen specific manner, suggesting that there was correct pairing of the introduced TCR genes. Tetramer binding does not always correlate with function: T cells which do not bind tetramer, but are still functional, have been described in the literature, as have cells which bind tetramer, but which do not secrete cytokines (186;218). Furthermore, it was observed with both the F5 and the WT1 TCRs that tetramer binding also increased in cells co-transduced with TCR and CD3ζ-GFP, which expressed higher levels of TCR, despite the Vβ expression being similar to that seen in TCR only or TCR + GFP transduced cells. This is likely to relate to there being a minimum density of TCR expression which is required in order for tetramer to stably bind. In the presence of additional CD3, TCR density may increase beyond the threshold required for tetramer binding.

Nonetheless, with regard to cytokine production, there was consistently a greater response seen in the TCR+CD3ζ-GFP cells compared to the TCR+GFP control cells expressing a similar level of Vβ2.1. However, it was difficult to detect a difference in avidity using ELISA on bulk transduced cells because the proportion of TCR/CD3 bright cells is small, and any effect may be diluted by cells which were only transduced with the TCR. However, intracellular cytokine staining allowed me to gate on the GFP bright cells, and compare IFNγ production by GFP bright CD8+ cells in the TCR+CD3ζ-GFP and TCR+GFP control populations, thus excluding from the analysis cells which expressed the TCR, but not additional CD3. This technique enabled me to demonstrate that increased density of TCR expression correlated with an increase in antigen specific IFNγ production as well as increased sensitivity: WT1 TCR+CD3ζ-GFP transduced cells not only produced more IFNγ at each peptide concentration, but were also able to recognise a ten fold lower concentration of pWT126 than WT1 TCR+GFP transduced cells (Fig 5.8). Analysis of the MFI of IFNγ

staining in WT1 TCR+ CD3ζ-GFP and WT1 TCR+GFP transduced cells was similar (4149 and 4420, respectively). This suggests that IFNγ production by individual cells following TCR triggering is not different. Rather, that the increased IFNγ production is a result of a greater proportion of the WT1 TCR+CD3ζ-GFP cells responding. Given that this is not due to differences in overall Vβ2.1 expression between the two groups, this suggest that increased density of Vβ2.1 expression in the CD3 high cells reaches a threshold above which they can respond to antigen.

My data is again in keeping with the work by Hofmann *et al,* who demonstrated that a five fold difference in TCR expression level was sufficient to see a difference in sensitivity: TCR high splenocytes were able to produce cytokine and lyse specific targets at a ten-fold lower peptide concentration (212). This work was somewhat at odds with previous data from Mathis' group, who had used a tetracyline based control system to generate transgenic mice expressing different levels of the same TCR. They showed that a 20 fold reduction in TCR expression, with T cells expressing as few as 1000 TCRs per CD8 T cell, could still result in cytokine production in response to saturating concentrations of antigen, although they did not address the issue of sensitivity (219). However, both of these models employ transgenic mice which only express a single TCR, so the data does not mimic the situation in TCR transduced primary T cells, where there is competition between the endogenous and exogenous TCRs for surface expression. The data presented in this chapter demonstrates that increasing the density of exogenous TCR expression, by co-transduction with CD3, can increase the T cell avidity and improve antigen specific function. This may be a strategy by which the avidity of a lower affinity TCR can be improved. There is now evidence that exogenous TCR expression following gene transfer can vary widely, depending on the characteristics - and the relative strength - of both the endogenous and exogenous TCR (220;221). It may be that 'stronger' TCRs, which fold and form complexes with CD3 molecules more efficiently than endogenous TCRs, will benefit most from the provision of additional CD3. In cases where the exogenous TCR naturally out-competes the endogenous TCR for CD3, the increased provision of CD3 molecules may further increase exogenous TCR density to a level which results in increased sensitivity.

A potential safety issue with retroviral TCR gene transfer is that it has been demonstrated that exogenous TCRs can mispair with endogenous TCR chains (150). Mispaired TCRs, comprised of α and β chains from each of the endogenous and exogenous TCRs, have not undergone negative selection in the thymus and

therefore have the potential to be autoreactive. Even if mispaired TCRs are not autoreactive, any mispairing may reduce the expression density and hence the efficacy of the desired TCR. A number of strategies have recently been employed to address this issue. TCRs have been engineered to include an additional cysteine residue in the constant regions of the α and β chains, resulting in the formation of a second disulphide bond between them. T cells transduced with cysteine-modified receptors showed increased tetramer binding, secreted more cytokine and showed increased antigen specific lysis when co-cultured with specific tumour cell lines, compared with T cells expressing wild type TCR (150;222). Hybrid TCRs have also been designed to incorporate murine constant regions and human variable regions. These hybrid TCRs show reduced mispairing with fully human TCRs when introduced into human T cells, combined with superior cell surface expression and biological activity (150;223). However, there is a possibility that a human host will mount an immune response against the murine component of such a TCR. In the same way as murine monoclonal antibodies have become increasingly humanised for clinical use, it is likely that murinization of the TCR constant region will be minimized to reduce its immunogenicity, if this strategy is to be used in a clinical setting.

An important question to address will be whether co-transduction of TCR and CD3 genes also results in increased expression of mispaired TCRs. The data presented here suggests that that co transfer of TCR and CD3 genes resulted in cells which expressed very high levels of the introduced TCR, but substantially reduced levels of endogenous Vα2 (Fig 5.10). Of note, the data presented in this chapter only assesses the effect of WT1 TCR expression on endogenous Vα2 expression, and it would be important to confirm this observation by repeating the experiment using antibodies against a range of different endogenous Vα and Vβ chains.

Recent data has shown that 'strong' TCRs are expressed at high levels following retroviral gene transfer, whereas 'weak' TCRs compete poorly with the endogenous TCR repertoire for CD3 molecules and are poorly expressed (220;224). Strategies such as murinisation of constant domains and cysteine modification of TCR chains also serve to increase the 'strength' of TCR expression (150;221;225). Since it is likely that co-transduction with CD3 will be most effective at increasing the expression of strong TCRs, there is the potential for these strategies to be used in combination. If cells expressing high levels of the exogenous TCR/CD3 consistently demonstrated decreased expression of endogenous TCR chains, these cells could

be sorted by FACS to produce a population of cells with both a low risk of mispairing which also express a high avidity exogenous TCR. Using these cells for adoptive transfer would reduce the risk of autoimmunity resulting from mispaired TCR chains. Furthermore, I would hypothesize that the adoptive transfer of a smaller number of higher avidity T cells, demonstrating increased sensitivity *in vitro*, would be required to confer tumour protection *in vivo*. It would be interesting to compare the ability of the TCR/CD3ζ-GFP high and TCR/GFP high cells to persist following adoptive transfer, as well as their ability to respond to antigen and confer tumour protection *in vivo*.

6. General Discussion

There are a number of potential barriers to successful cancer immunotherapy. The majority of targets identified to date are over-expressed self antigens, such as WT1. This raises the issue of immunological tolerance, as one would expect CTL which recognise self antigens with high avidity to be deleted in the thymus. However, central tolerance is incomplete, and it is clear that autologous responses against tumour antigens such as WT1 can be detected in patients with cancer (67;116;157;160;184). Nonetheless, the impact of autologous responses on the natural history of tumour development is unknown. It may be that autologous immune responses against tumour antigens are evidence of immune surveillance. However, an alternative explanation is that they are just evidence of the presence of the tumour, since recent data in a sporadic murine tumour model demonstrated that the appearance of tumour specific antibody and CD8 T cell responses correlated with the development of tumours, but not with the emergence of tumour escape variants (12).

It has been demonstrated in murine models and in clinical trials that autologous responses against WT1 can be boosted by vaccination. Different groups have shown that vaccination of mice with WT1 peptides can generate WT1 specific T cells capable of lysing target cells loaded with their cognate peptide. However, whereas Oka *et al* demonstrated that these T cells *could* protect mice against subsequent challenge with WT1 expressing tumour cells, this was inconsistent with work by Ramirez *et al*, who subsequently showed that WT1 CTL generated by vaccination did not kill tumour cells in vitro or confer tumour protection in vivo (93;117). What is not clear is whether this discrepancy is due to vaccination having induced WT1 CTL of different avidities in the two models, or whether it is a result of having used different WT1 expressing tumours as targets. Both Phase I WT1 vaccination trials in humans have demonstrated that vaccination resulted in an increased frequency of WT1 CTL in a proportion of patients with WT1 expressing malignancies. Furthermore, the presence of WT1 CTL was associated with clinical responses including a reduction in leukemic blast cells, tumour markers, WT1 mRNA copy number or tumour size (116) (188). However, these results need to be interpreted with caution owing to the small number of patients enrolled in these trials, and it should be noted that only 3 out of 5 patients with solid tumours would be considered to have shown a partial response to treatment according to the standard RECIST criteria used in oncological clinical trials (226). In the absence of data from a large, randomised, Phase III clinical trial, a question mark still remains over whether or not WT1 CTL generated from the

autologous repertoire of cancer patients will be of sufficient avidity to kill tumour cells in vivo. Tumour vaccination strategies in general have not been met with the large scale clinical success that was initially anticipated. Most reports are from small phase I trials and results are varied, with some studies demonstrating a correlation between clinical responses and vaccine induced immune responses, and others showing immune responses in the absence of clinical responses, and vice versa (227-229). The vast majority of clinical work in the vaccination field has been performed in melanoma, where a recent review incorporating data from 422 patients with metastatic melanoma who underwent peptide vaccination demonstrated an objective clinical response rate of only 2.6% when RECIST criteria were applied (89).

At present there is a lack of compelling evidence that vaccination in isolation will be enough to cause regression of solid tumours in the majority of patients. Concerns remain over whether tumour antigen specific T cells from the autologous repertoire will be of sufficient avidity to mount an effective anti tumour response in vivo. Recent data in a murine transgenic model of T cell tolerance demonstrated that although T cells specific for the murine double minute tumour antigen escaped central tolerance and were present in the periphery, their ability to proliferate and express effector molecules in response to antigen stimulation was impaired. These defects could be restored if cells were stimulated in the presence of IL2, and to a lesser extent IL15 or IL21. However, even after tolerance was broken with IL2, the 'rescued' T cells were of lower avidity than non tolerant control T cells expressing the same TCR (230). Thus, even if tolerance can be broken and autologous WT1 specific T cell responses can be boosted by vaccination, whether these cells will be of sufficient avidity to lyse tumour cells in vivo is not clear at present, but appears doubtful.

TCR gene therapy is a strategy by which autologous T cells which recognise tumour associated antigens with high avidity can be generated. This technique enables the production of large numbers of autologous, antigen specific T cells, with the added advantage that the introduced TCR specificity can be directed against poorly immunogenic targets. There are several methods by which high avidity CTL specific for TAAs can be generated. Immunisation of HLA transgenic mice, the allo-restricted approach, in vitro mutagenesis of TCRs and in vitro selection using phage display can be used to generate TCRs with increased peptide/MHC binding affinity (133;164;231-233) (133;234-236). TCRs can be isolated and functionally tested before being retrovirally transferred into patients' T cells, where the redirected T cells demonstrate the same functional avidity as the original parent T cell from which the

TCR genes are cloned (133;237). Transduced T cells have been shown to contribute to tumour clearance in murine models (202) (200) and the first clinical trial of TCR gene transfer was recently reported. Retroviral gene transfer was used to transduce peripheral blood lymphocytes, taken from patients with melanoma, with the genes encoding the α and β chains of a TCR with specificity for a MART 1 peptide presented by HLA-A*0201 (238). The 17 patients in the gene transfer study were lymphodepleted prior to receiving autologous T cells transduced with the MART-1 TCR. The engineered T cells persisted in 15 patients, and the two patients with the highest levels of circulating anti melanoma T cells showed objective regression of metastatic lesions and remained in remission 18 months after treatment (137). The results of this study prove that retroviral TCR gene transfer can be used to confer anti tumour specificity upon a large number of T cells, and that these T cells can engraft in patients and persist at high levels longterm.

While TCR gene transfer holds promise, there may yet be obstacles to overcome with respect to either the safety or the efficacy of this strategy. Firstly, mispairing of endogenous and exogenous TCR chains may result in 'off target' toxicity. Secondly, high avidity CTL may cause 'on target' toxicity by attacking normal tissues which express low levels of the target antigen. Thirdly, adoptively transferred T cells may be subject to tolerance mechanism which render them unresponsive in vivo. I will address each of these in turn.

Although there is no evidence of off target toxicity in murine models to date, it has been demonstrated that exogenous TCR are able to mispair with endogenous TCR chains, resulting in the expression of TCRs which have not undergone thymic education, with unpredictable specificities, which have the potential to be autoreactive. As described in chapter 5, several strategies have been employed to reduce mispairing and increase expression of the desired TCR. These include murinization of TCR constant domains and cysteine modified TCR chains, both of which have been shown to result in increased TCR expression and tetramer binding compared with wild type TCRs (150; 222; 225). Furthermore, the strategy described in Chapter 5 of this thesis may be a mechanism by which cells expressing only high levels of the introduced TCR can be generated. Research is ongoing into alternative means by which the risk of mispairing may be reduced. It has recently been shown that TCR α and β chains which have each been linked to a CD3ζ chain did not mispair with endogenous TCR chains in a Jurkat T cell model (239). Since γδ TCR chains cannot mispair with αβ TCR chains, transferring αβ TCR chains into γδ T cells

should not result in any mispairing, and has previously been shown to result in the expression of exogenous αβ TCR which produce cytokine and lyse target cells in an antigen specific manner (240). Transduction of viral specific T cells is a strategy by which the potential number of mixed dimers can be reduced, since anti viral responses consist of T cells with a restricted TCR repertoire (201). An alternative approach would be to transduce haematopoetic stem cells (HSC) with TCR genes. In vitro generation of mature, antigen specific T cells by TCR gene transfer into thymus or cord derived HSC has recently been reported (241;242). Allelic exclusion of the endogenous TCR β chain meant that mixed dimer formation was reduced, but not entirely avoided due to some endogenous α chain expression. Furthermore, while the risk of transformation of mature T cells is low, the risk in HSC may be higher, making this a less appealing strategy (243). In a clinical trial of X linked severe combined immunodeficiency disease, 4 children treated with HSC retrovirally transduced with the common γ chain developed T lymphoproliferative disorders. This was later found to be secondary to retroviral insertion into the LMO-2 oncogene intron on chromosome 11, with subsequent upregulation (244-246). Although there is no evidence to date of transformation of mature T cells with retroviral vectors, the use of lentiviral vectors is also being investigated, since it has been shown that lentiviral vectors insert near promoters at a lower frequency.

While there is a concern that low avidity, TAA specific CTL from the autologous repertoire may not be efficacious, high avidity, self reactive CTL may pose the opposite problem. The majority of targets for tumour immunotherapy are over-expressed self proteins, so there is a risk that targeting TAA may result in autoimmune damage. In murine models and in clinical trials it has been demonstrated that the successful induction of CTL responses against melanoma TAAs (such as melan A or gp100) has been associated with the development of vitiligo (247-250). T cell therapies targeting TAAs with a more ubiquitous distribution have not been studied in the same detail as yet, although it has been shown that high avidity p53 specific CTLs (generated in $p53^{-/-}$ transgenic mice) can provide tumour protection without causing autoimmune damage in mice (251). A moderately high affinity TCR was used in the TCR gene therapy trial, and work is now ongoing by the same group to test a higher avidity TCR. It remains to be seen whether any morbidity resulting from autoimmune disease outweighs the associated anti tumour benefit. A balance needs to be struck between TAA specific CTL which are of high enough avidity to mediate tumour killing, but which do not cause significant autoimmune damage to healthy tissue. It is likely that a large discrepancy between the expression

level of the target antigen on tumour tissue compared to that on normal tissue will be an important factor in this regard, as will the pattern of distribution of the TAA in normal tissue. A validated definition of WT1 over-expression, which predicts tumour susceptibility to adoptively transferred TCR transduced cells, may be required.

An alternative consequence of TCR gene transfer may be that T cells expressing high avidity TCRs will be more susceptible to tolerance mechanisms in vivo following adoptive transfer. At present it is unclear why only 2 patients in the TCR gene therapy trial responded clinically, when 15 patients demonstrated persistence of the engineered T cells, which made up at least 10% of their circulating T cells for more than two months after treatment. One explanation is that the adoptively transferred T cells were rendered unresponsive in vivo. There may therefore be a role for combining adoptive T cell transfer with mechanisms to break tolerance. The conditions required for induction of tolerance are not well defined, but data from animal models suggests that the presentation of antigen in the absence of inflammatory stimuli results in tolerance (252). There are a number of mechanisms of T cell tolerance, including down regulation of the TCR or other molecules such as CD8, and interference with the IL2 signalling pathway (253-256). Various mechanisms by which tolerance can be reversed have been identified, most of which involve manipulation of T cell co-stimulatory pathways. For example, exogenous IL2, signalling through OX40 (CD134), CD40 ligation and anti CTLA-4 antibodies have all been shown to break tolerance in murine models, and the data presented in Chapter 3 is the first demonstration of tolerance reversal with IL15 in human T cells (159;230;257-259). However, in addition to naturally occurring tolerance mechanisms, there are additional factors to consider in the presence of solid tumours. It has been demonstrated both in humans and in murine tumour models that T cells derived from tumour bearing hosts are dysfunctional (130, 156). As described in Chapter 4, chronic inflammation and persistent IFNγ production may result in the generation of myeloid suppressor cells, which contribute towards T cell dysfunction via downregulation of the CD3ζ chain. In addition, tumours may actively secrete immunosuppressive factors such as TGFβ or IL10. The full range of mechanisms by which tumours induce tolerance are still undefined, and the roles played by regulatory T cells, myeloid suppressor cells and other immunosuppressive factors present in the tumour microenvironment continue to emerge.

Tolerance induction is only one possible explanation as to why only 2 out of 15 patients in the trial responded clinically to TCR gene therapy. It may be that the level

of expression of the target antigen on tumours varied between patients, or that the tumours which did not respond had downregulated MHC Class 1 expression or become otherwise immunoresistant. The factors that allow certain patients to respond well to immunotherapy need to be more fully understood, in order that treatments can be improved and also targeted to those patients most likely to benefit. Given the ability of tumours to mutate and react to their environment, there is also a concern that therapy directed at a single target, such as WT1, may apply selective pressure to the tumour and lead to the emergence of escape variants. Selecting a target which is required for maintenance of the oncogenic phenotype will hopefully mean that down-regulation of the target will limit the cell's malignant potential. Another option is to simultaneously attack more than one target, via more than one mechanism. Combination therapy has previously been shown to be effective in both antiretroviral therapy as well as chemotherapy, and trials which combine standard chemotherapy with immunotherapy, or immunotherapy with tyrosine kinase inhibitors, are currently underway. Although B cell cancer immunotherapy has seen greater clinical acclaim in recent years through the success of monoclonal antibody therapy, TCR gene transfer has now entered Phase I clinical trials. Further work is needed to identify the patients most likely to benefit from such a strategy, and randomised trial data is still some way off, but the field of T cell cancer immunotherapy is currently one to be watched closely.

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Appendix I:

CD3 *zeta*-F2A-CD3 *epsilon*-T2A-CD3 *delta* -E2A-CD3 *gamma* nucleotide sequence

5' Not 1 and 3' Sal1 restriction sites are underlined
2A sequences are in **BOLD CAPS**

Gcggccgc**atg**aagtggaaagtgtctgttctcgcctgcatcctccacgtgcggttcccaggagcagaggcacagag
ctttggtctgctggatcccaaactctgctacttgctagatggaatcctcttcatctacggagtcatcatcacagccctgtacc
tgagagcaaaattcagcaggagtgcagagactgctgccaacctgcaggaccccaaccagctctacaatgagctca
atctagggcgaagagaggaatatgacgtcttggagaagaagcgggctcgggatccagagatgggaggcaaacag
cagaggaggaggaacccccaggaaggcgtatacaatgcactgcagaaagacaagatggcagaagcctacagtg
agatcggcacaaaaggcgagaggcggagaggcaaggggcacgatggcctttaccagggtctcagcactgccac
caaggacacctgtgatgccctgcatatgcagaccctggcccctcgc**GTGAAGCAGACTTTGAATTTTG**
ACCTTCTCAAGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCCatg**cggtggaa**
cactttctggggcatcctgtgcctcagcctcctagctgttggcacttgccaggacgatgccgagaacattgaatacaaa
gtctccatctcaggaaccagtgtagagttgacgtgccctctagacagtgacgagaacttaaaatgggaaaaaaatgg
ccaagagctgcctcagaagcatgataagcacctggtgctccaggatttctcggaagtcgaggacagtggctactacg
tctgctacacaccagcctcaaataaaaacacgtacttgtacctgaaagctcgagtgtgtgagtactgtgtggaggtgga
cctgacagcagtagccataatcatcattgttgacatctgtatcactctgggcttgctgatggtcatttattactggagcaag
aataggaaggccaaggccaagcctgtgacccgaggaaccggtgctggtagcaggcccagagggcaaaacaag
gagcggccaccacctgttcccaacccagactatgagcccatccgcaaaggccagcgggacctgtattctggcctga
atcagagagcagtc**GAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAG**
AATCCTG*G*CCCAatggaacacagcgggattctggctagtctgatactgattgctgttctcccccaagggagcc
ccttcaaggtacaagtgaccgaatatgaggacaaagtatttgtgacctgcaataccagcgtcatgcatctagatggaa
cggtggaaggatggtttgcaaagaataaaacactcaacttgggcaaaggcgttctggacccacgagggatatatctg
tgtaatgggacagagcagctggcaaaggtggtgtcttctgtgcaagtccattaccgaatgtgccagaactgtgtggag
ctagactcgggcaccatggctggtgtcatcttcattgacctcatcgcaactctgctcctggctttgggcgtctactgctttgc
aggacatgagaccggaaggccttctggggctgctgaggttcaagcactgctgaagaatgagcagctgtatcagcctc
ttcgagatcgtgaagatacccagtacagccgtcttggagggaactggccccggaacaagaaatct**CAATGTAC**
TAACTACGCTTTGTTGAAACTCGCTGGCGATGTTGAAAGTAACCCCGGTCCTat
ggagcagaggaagggtctggctggcctcttcctggtgatctctcttcttcaaggcactgtagcccagacaaataaagc
aaagaatttggtacaagtggatggcagccgaggagacggttctgtacttctgacttgtggcttgactgacaagactatc
aagtggcttaaagacgggagcataataagtcctctaaatgcaactaaaaacacatggaatctgggcaacaatgcca
aagaccctcgaggcacgtatcagtgtcaaggagcaaaggagacatcaaacccctgcaagtgtattacagaatgtg
tgaaaactgcattgagctaaacataggcaccatatccggctttatcttcgctgaggtcatcagcatcttcttccttgctcttg
gtgtatatctcattgcgggacaggatggagttcgccagtcaagagcttcagacaagcagactctgttgcaaaatgaac

agctgtaccagcccctcaaggaccgggaatatgaccagtacagccatctccaaggaaaccaactgaggaagaag
gtcgac

Appendix II: NOT1—Sal1–IRES GFP– LPRE pMP71

Not1 (red)
Cloning site underlined
Sal1 (blue)

GCGGCCGC____GTCGACCTC GAGATCCGCC CCTCTCCCTC
CCCCCCCCCT
AACGTTACTG GCCGAAGCCG CTTGGAATAA GGCCGGTGTG CGTTTGTCTA
TATGTTATTT TCCACCATAT TGCCGTCTTT TGGCAATGTG AGGGCCCGGA
AACCTGGCCC TGTCTTCTTG ACGAGCATTC CTAGGGGTCT TTCCCCTCTC
GCCAAAGGAA TGCAAGGTCT GTTGAATGTC GTGAAGGAAG CAGTTCCTCT
GGAAGCTTCT TGAAGACAAA CAACGTCTGT AGCGACCCTT TGCAGGCAGC
GGAACCCCCC ACCTGGCGAC AGGTGCCTCT GCGGCCAAAA GCCACGTGTA
TAAGATACAC CTGCAAAGGC GGCACAACCC CAGTGCCACG TTGTGAGTTG
GATAGTTGTG GAAAGAGTCA AATGGCTCTC CTCAAGCGTA TTCAACAAGG
GGCTGAAGGA TGCCCAGAAG GTACCCCATT GTATGGGATC TGATCTGGGG
CCTCGGTGCA CATGCTTTAC ATGTGTTTAG TCGAGGTTAA AAAAACGTCT
AGGCCCCCCG AACCACGGGG ACGTGGTTTT CCTTTGAAAA ACACGATGAT
AATATGGCCA CAACCATGGT GAGCAAGGGC GAGGAGCTGT TCACCGGGGT
GGTGCCCATC CTGGTCGAGC TGGACGGCGA CGTAAACGGC CACAAGTTCA
GCGTGTCCGG CGAGGGCGAG GGCGATGCCA CCTACGGCAA GCTGACCCTG
AAGTTCATCT GCACCACCGG CAAGCTGCCC GTGCCCTGGC CCACCCTCGT
GACCACCCTG ACCTACGGCG TGCAGTGCTT CAGCCGCTAC CCCGACCACA
TGAAGCAGCA CGACTTCTTC AAGTCCGCCA TGCCCGAAGG CTACGTCCAG
GAGCGCACCA TCTTCTTCAA GGACGACGGC AACTACAAGA CCCGCGCCGA
GGTGAAGTTC GAGGGCGACA CCCTGGTGAA CCGCATCGAG CTGAAGGGCA
TCGACTTCAA GGAGGACGGC AACATCCTGG GGCACAAGCT GGAGTACAAC
TACAACAGCC ACAACGTCTA TATCATGGCC GACAAGCAGA AGAACGGCAT
CAAGGTGAAC TTCAAGATCC GCCACAACAT CGAGGACGGC AGCGTGCAGC
TCGCCGACCA CTACCAGCAG AACACCCCCA TCGGCGACGG CCCCGTGCTG
CTGCCCGACA ACCACTACCT GAGCACCCAG TCCGCCCTGA GCAAAGACCC
CAACGAGAAG CGCGATCACA TGGTCCTGCT GGAGTTCGTG ACCGCCGCCG
GGATCACTCT CGGCATGGAC GAGCTGTACA GAATTCGAGC ATCTTACCGC
CATTTATTCC CATATTTGTT CTGTTTTTCT TGATTTGGGT ATACATTTAA
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GTAATTACTA GTTCAGGTGT ATTGCCACAA GACAAACATG TTAAGAAACT
TTCCCGTTAT TTACGCTCTG TTCCTGTTAA TCAACCTCTG GATTACAAAA
TTTGTGAAAG ATTGACTGAT ATTCTTAACT ATGTTGCTCC TTTTACGCTG
TGTGGATATG CTGCTTTAAT GCCTCTGTAT CATGCTATTG CTTCCCGTAC
GGCTTTCGTT TTCTCCTCCT TGTATAAATC CTGGTTGCTG TCTCTTTATG
AGGAGTTGTG GCCCGTTGTC CGTCAACGTG GCGTGGTGTG CTCTGTGTTT
GCTGACGCAA CCCCCACTGG CTGGGGCATT GCCACCACCT GTCAACTCCT
TTCTGGGACT TTCGCTTTCC CCCTCCCGAT CGCCACGGCA GAACTCATCG
CCGCCTGCCT TGCCCGCTGC TGGACAGGGG CTAGGTTGCT GGGCACTGAT
AATTCCGTGG TGTTGTCGGG GAAGCTGACG TCCTTTCCAT GGCTGCTCGC
CTGTGTTGCC AACTGGATCC TGCGCGGGAC GTCCTTCTGC TACGTCCCTT
CGGCTCTCAA TCCAGCGGAC CTCCCTTCCC GAGGCCTTCT GCCGGTTCTG
CGGCCTCTCC CGCGTCTTCG CTTTCGGCCT CCGACGAGTC GGATCTCCCT
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GGTCGTCACC TGTGCAGAAT TGCGAACCAT GGATTCCACC GTGAACTTTG

TCTCCTGGCA TGCAAATCGT CAACTTGGCA TGCCAAGAAT TCGGATCCAA
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TGTTCCCTAA GTCCAACTAC TAAACTGGGG GATATTATGA AGGGCCTTGA
GCATCTGGAT TCTGCCTAGC GCTAAGCTTC CTAACACGAG CCATAGATAG
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TGCATCCGAA TCGTGGACTC GCTGATCCTT GGGAGGGTCT CCTCAGATTG
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CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG
AGAGGCGGTT TGCGTATTGG GCGCTCTTCC GCTTCCTCGC TCACTGACTC
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TACCGGATAC CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC
AATGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG
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CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC
TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT
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AGACACTTCC CGCCCCCGTC TGAATTTTTG CTTTCGGTTT TACGCCGAAA
CCGCGCCGCG CGTCTTGTCT GCTGCAGCAT CGTTCTGTGT TGTCTCTGTC
TGACTGTGTT TCTGTATTTG TCTGAAAATT AGCTCGACAA AGTTAAGTAA
TAGTCCCTCT CTCCAAGCTC ACTTACAG